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Characterizing the Epidemiology of Bluetongue and Epizootic Hemorrhagic Disease Virus in Louisiana

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CHARACTERIZING THE EPIDEMIOLOGY OF BLUETONGUE AND EPIZOOTIC HEMORRHAGIC DISEASE VIRUS IN LOUISIANA

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ABSTRACT

Bluetongue virus (BTV) and Epizootic Hemorrhagic Disease Virus (EHDV) are arthropod-borne viruses (Reoviridae; *Orbivirus*) transmitted by biting midges in the genus *Culicoides*. These viruses cause hemorrhagic disease and induce serious morbidity and mortality rates in wild and captive deer. Cattle are normally asymptomatic but serve as the reservoir/amplifying host for these viruses and play an important role in the transmission cycle. The objectives of this study were to characterize the epidemiology of these viruses as they relate to white-tailed deer and cattle, evaluate different trap types for collecting *Culicoides* midges, and determine if these viruses can be detected in bone marrow of white-tailed deer. Using CDC black light traps baited with dry ice, we identified 15 species of *Culicoides* during a 7 year study at the Idlewild Research Station in Clinton, La which experienced several outbreaks of hemorrhagic disease in the white-tailed deer herd. Real-time quantitative PCR was performed to screen for BTV and EHDV in 1711 pools representing 24,859 specimens collected at the farm. The results showed 5 species (*C. debilipalpis, C.stellifer, C. venustus, C. haematopotus, crepuscularis*) to be positive for BTV and EHDV. The overall results showed a distinct positive correlation with collected specimens of *C. debilipalpis* and *C. stellifer* and the number of BTV/EHDV confirmed deer deaths. These 2 species accounted for over 60% of BTV positive pools and supports evidence that they are the probable BTV vectors in Louisiana. This was the first report of EHDV PCR positive field collected specimens from these species in Louisiana. Agar gel immunodiffusion test results showed a significant increase in BTV/EHDV antibodies in cattle and white-tailed deer from before the vector season versus after the season for each year. Notably, PCR results showed

cows and deer to be positive for EHDV-6 and BTV-12, which was the first time these serotypes have been reported in Louisiana. We compared the number of specimens and species captured using 3 animal baited traps, CDC traps with and without black light, and a rotator trap which collected at different time periods. Animal-baited traps did not capture any different species of *Culicoides* than the CDC black light traps. The most active time period for *Culicoides* midges was 0600-0800h in which most specimens were captured, especially for *C. debilipalpis*. The minimum infection rate for BTV was higher in midges collected in traps without light but not for EHDV. Bone marrow samples collected from white-tailed deer were PCR positive up to 4 months postmortem for BTV and 3 months for EHDV. This can be a useful tool for determining serotype and probable cause of death during epizootics, especially for wildlife agents and deer farmers.

INTRODUCTION

Bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV) are in the genus *orbivirus,* family Reoviridae and are transmitted to ruminants by infected *Culicoides* midges (Tabachnick 1996). The worldwide distribution of these viruses is determined by the detection of antibodies or clinical disease in ruminants which are the susceptible host for BTV and EHDV infections. In cattle, infections of BTV and EHDV are normally subclinical and no symptoms occur, but they do incur long lasting viremia and are considered reservoir hosts for these viruses. In domestic ruminants BTV causes bluetongue disease (BT) and EHDV causes epizootic hemorrhagic disease (EHD); in wild ruminants, the term for BTV or EHDV infections is hemorrhagic disease (HD). The highest mortality and morbidity caused by orbivirus infections is observed in white-tailed deer and sheep (Tabachnick 1996). There are 26 recognized serotypes of BTV and 7 EHDV serotypes worldwide which are transmitted by different vector species of *Culcoides* (Maan et al. 2012).

The only known vectors of BTV and EHDV are biting midges of the genus *Culicoides*, family Ceratopogonidae. The primary vector for BTV and EHDV in the U.S is *C. sonorensis,* although *C. insignis* is also a confirmed vector of BTV in Florida (Tanya et al. 1992). However, there have been numerous field studies with reported transmission of BTV and EHDV when *C. sonorensis* and *C. insignis* were rare or absent. Becker et al. (2010) collected specimens from 10 species of *Culicoides* in Louisiana over a 2 year period in an area with BTV transmission of which 4 pools of flies (*C. crepuscularis, C. debilipalpis, C. haematopotus,* and *C. furens*) were PCR positive for BTV; no specimens of *C. sonorensis* were captured throughout the study. More recently, McGregor et al. (2018) have implicated *C. venustus* and *C. stellifer* as vectors for EHDV

after detecting positive pools from field-collected specimens during an epizootic of EHD at white-tailed deer farms in Florida where *C. sonorensis* was absent. Therefore, it is important to determine the local species of *Culicoides* in areas with BTV/EHDV transmission.

We conducted a trap type study comparing and contrasting different methods of trapping *Culicoides* midges which included animal baited traps, CDC suction traps with and without light, and a rotator trap which collected at different time periods throughout the night. We also set up a 7 year prospective study recording seasonal entomological data from *Culicoides* midges using different traps and serological and virus diagnostic data from tissues/blood samples from cattle and white-tailed deer at the Bob R. Jones Idlewild research station. We were able to observe peaks of activity for midges with confirmed orbivirus white-tailed deer deaths. The virus serotype was determined from cattle and blood samples. In addition, we conducted a study to determine the duration of RNA presence for BTV/EHDV in the bone marrow of white-tailed deer post mortem. We had the unique opportunity to work at a research station which houses penned white-tailed deer, pastured cattle, and naturally occurring *Culicoides* midges and were able to compile a distinctive data set that will be highly useful for studying the epidemiology of BTV and EHDV and hopefully fill in research gaps which are lacking. This research is especially relevant for reducing hemorrhagic diseases caused by BTV and EHDV in wild and farmed white-tailed deer.

CHAPTER 1. LITERATURE REVIEW

1.1 Biology and Ecology of Ceratopogonidae

Over 5500 species in 125 genera have been described and placed in the family Ceratopogonidae, order Diptera. The size of these flies ranges from 1- 4mm and they are considered to be some of the smallest flies in the world. Only four genera of ceratopogonids are known to contain hematophagous, or blood feeding, adult females: *Austroconops, Culicoides, Forcipoymia* subgenus *Lasiohelea,* and *Leptoconops* (Mellor et al. 2000). Ceratopogonids are found throughout the world in association with various aquatic or semiaquatic habitats. Some common names of ceratopogonids include sand flies, punkies, no-see-ums, midges, and biting midges (Mellor et al. 2000). In south Louisiana, they are known as and commonly referred to as gnats.

The life cycle of ceratopogonids is holometabolous which includes the egg, four larval stages, pupa, and adult. Adult females usually lay eggs in batches that hatch within two to seven days and are not resistant to drying (Meiswinkel et al. 1994). Larvae require a certain amount of moisture and can be found in a wide range of habitats; common examples include edges of pools, streams, marshes, bogs, beaches, swamps, tree holes, irrigation pipe leaks, animal dung, and rotting fruit (Blanton and Wirth 1979). The development of larvae differs among species and the duration of each stage is dependent upon temperature, humidity, and precipitation. The larvae of some species are predacious, feeding on nematodes, protozoans, immature insects, and other small aquatic organisms (Mullen 2002). Other species have larvae that can feed on particles of vegetable matter to gain necessary nutrients. The overwintering stage for most species is the fourth-instar larvae in diapause (Kettle 1984). However, adults of some species are capable of overwintering, especially in areas with mild winters (Garry and Mullens 2000:

Khalaf 1969). Pupae are usually free floating but sometimes are loosely attached to debris and the pupal stage usually lasts for two to three days, but can last for several weeks depending upon species and temperature.

Most hematophagous female ceratopogonids are nocturnal but some species are diurnal. Specimens of *Culicoides paraensis* Goeldi are known to bite people during the day, and some species in the genus *Austroconops* are day biters (Borkent and Craig 2004; Pinheiro et al. 1981). For most female *Culicoides*, a blood meal is required for egg production. One study showed that *C. variipennis* Coquillett takes a blood meal of 0.56 mg, which is about 20% the size of a blood meal of a mosquito (Tempelis and Nelson 1971). There are autogenous ceratopogonid species; the nutrition to produce eggs is provided by energy reserves obtained as larvae (Blanton and Wirth 1979). Linley (1983) reported that 38 species of Ceratopogonidae are autogenous, including *C. furens* Poey. Male ceratopogonids feed exclusively on carbohydrates, such as plant sugars, and do not have blood-feeding mouthparts.

Adult biting midges are usually short-lived and live no longer than ten days, but there are some exceptions with individuals living more than 90 days. Cribb (2000) showed that specimens of the genus *Forcipomyia* can live up to 39 days after collection. Goffredo (2004) reported that out of 1,500 wild-caught midges from the *C. obsoletus* complex, 3 lived for 92 days in the laboratory. Females that take more than one blood meal are very important because female flies must take at least two blood meals to transmit pathogens that are not vertically transmitted. However, in nature only a very small percentage of female ceratopogonid populations are successful at getting a second blood meal (Mullen 2002).

1.2 Ceratopogonids as Vectors of Disease

There are more than 1,400 species of *Culicoides* of which 96% are obligate blood-feeders that attack birds and mammals (Meiswinkel et al. 1994). Specimens of the genus *Culicoides* can transmit pathogens to birds, humans, and animals and compromise the most important genus of the family Ceratopogonidae. Out of the 125 genera of Ceratopogonidae, only members of the genus *Culicoides* are known to be biological vectors of arboviruses. Worldwide more than 50 arboviruses have been isolated from *Culicoides* spp. (Mellor et al. 2000) and some of these viruses are significant agents that cause disease in humans and animals.

Bluetongue virus (BTV) is in the family Reoviridae, genus Orbivirus, and is the most important pathogen transmitted by members of the genus *Culicoides*. Bluetongue virus can affect all species of ruminants, and causes severe (often fatal) hemorrhagic disease in some species of sheep and deer. Mortality rates can reach 90% in infected whitetail deer herds and 70% in certain susceptible breeds of sheep (Center for Food Security and Public Health 2006). The largest known outbreak of bluetongue disease occurred between 1956-1960 when over 179,000 sheep died of BTV infection in Spain and Portugal (Gorman 1990). Bluetongue disease is classified as a List A disease by Office of International Epizootics because of the potential economic impact and ability for rapid spread. Members of the genus *Culicoides* are the only known competent vectors of BTV worldwide (Kramer et al. 1985); specimens from different species of *Culicoides* are the biological vectors of BTV around the world (Tabachnick 1996).

Epizootic hemorrhagic disease virus (EHDV) is also an orbivirus and is very similar to BTV and also causes epizootic hemorrhagic disease in ruminants. Epizootic hemorrhagic disease is considered to be the most important infectious disease of wild

deer in the U.S. (Nettles et al. 1991). The clinical signs in white-tailed deer are identical for BTV and EHDV and virus isolation studies or PCR are required to differentiate the two viruses. The only known vectors of EHDV are flies in the genus *Culicoides*. In the U.S., the primary vector of EHDV is considered to be *C. sonorensis* Wirth and Jones but other species of *Culicoides*, such as *C. lahillei* (= *C. debilipalpis* (Lutz)) have been suspected as vectors (Smith et al. 1996). More recent studies have implicated *C. stellifer* Coquillet and *C. venustus* Hoffman as vectors of EHDV (McGregor et al. 2019)

African horse sickness virus (AHDV) causes a serious, highly fatal disease in equids that can cause up to 90% mortality rates in horses (Mellor et al. 1993). The known vector of this virus is *C. imicola* Kieffer (Capela et al. 2003) but BTV has also been isolated from *C. tororoensis* Khamala & Kettle and *C. milnei* Glick (Walker and Davies 1971). The disease is mainly found in sub-Saharan Africa, but has occurred outside Africa on a few occasions, the most notable of which was a severe outbreak in the Near and Middle East from 1959-1963 (Lubroth 1988).

Vesicular stomatitis, caused by vesicular stomatitis virus (VSV), is an infectious viral disease that primarily affects cattle, horses, and swine and can have devastating effects on the U.S. cattle industry. Vesicular stomatitis also can affect humans, especially when handling animals infected with VSV (Mead et al. 2000). The transmission of the virus is not fully understood, but in the U.S., it has been shown to infect salivary glands of *C. sonorensis* and biting midges are considered as potential vectors of VSV (Drolet et al. 2005). Walton et al. (1987) were the first to report VSV from field-collected *Culicoides*; VSV was isolated from specimens of *C. stellifer, C. variipennis,* and *C. (selfia)* spp.

Oropouche virus is the most important known human pathogen that is transmitted by biting midges in the genus *Culicoides*. The virus causes a disease with similar symptoms to dengue fever in humans, and is transmitted by *C. paraensis* Goeldi. Oropouche fever occurs in the Amazonic region, Panama, and the Carribean and is considered a major public health problem (Mohammad et al. 2001; Yanase et al. 2005), causing more than half million cases in Brazil alone (Anderson 1961). Mercer et al. (2005) showed what was once thought to be one species in Peru was actually two different species, *C. paraensis* and *C. insinuates* Wirth and Blanton, and his findings helped explain why the distribution of human cases of Oropouche did not correspond to the assumed range of *C. paraensis*.

Protozoan parasites in the genus *Haemoproteus* are known to be transmitted by *Culicoides* midges (Levine 1961). The parasite *Haemoproteus danilewski* causes a malaria-like disease in populations of wild birds and can affect survival and reproduction of infected birds (Garvin et al. 2003). Garvin and Greiner (2003) conducted a 2 year survey on the seasonal abundance of *Culicoides* spp. in south Florida and experimentally challenged the most abundant ornithophilic species with *H. danilewskyi.* The authors found three species (*C. edeni* Wirth and Blanton, *C. knowltoni* Beck, and *C. arboricola* Root and Hoffman) capable of supporting sporogonic development of *H. danilewskyi* and suggested that *C. edeni* was the most important vector of *H. danilewski.*

A common filarial nematode of equines is *Onchocerca cervicalis*, which can cause severe dermatitis in horses (Foil *et. al* 1984; Rabalais et al. 1973; Stannard et al. 1975). The natural biological vectors of this filariid are biting midges in the genus *Culicoides* (Collins and Jones 1978). In the U.S. the primary vector is considered to be *C. sonorensis* (Foil et al. 1984) and the prevalence of equine onchocerciasis has been

reported as high as 82.6% in horses in the gulf coast regions of Louisiana and Mississippi (Klei et al. 1984). Foil et al. (1987) showed that *O. cervicalis* skin microfilariae of ponies and specimens of *C. sonorensis*, collected in light traps had corresponding peaks in south Louisiana.

1.3 Factors Affecting Ceratopogonids as Vectors of Disease

There is little to no evidence to suggest that any arbovirus can be vertically or venereally transmitted in species of *Culicoides*. White et al. (2005) did detect segments of BTV RNA in pools of larvae and pupae of both *C. sonorensis* and *C. crepuscularis* Malloch, but they did not isolate the virus and concluded that BTV was not vertically transmitted in the midges they tested. Therefore, to transmit BTV, female biting midges have to take a viremic blood meal from a vertebrate host and then bite another host after the virus has replicated and disseminated to the salivary glands (Mellor et al. 2000). In competent arbovirus vectors, virus particles attach to gut cells in the hind of the midgut and begin to replicate (Eaton et al. 1990). Virus particles then escape the midgut and enter the hemolymph where they infect secondary organs including the salivary glands. Virus particles then are released into the salivary ducts and are available for transmission during subsequent biting (Chandler et al. 1985; Fu et al. 1996). Most viral particles are transmitted when female midges are probing to find an efficient entry point on the host to obtain a blood meal. A number of factors must be considered when studying members of the genus *Culicoides* and their ability to transmit viruses.

Climate and weather can have a substantial impact on life cycles and populations of *Culicoides*, and therefore outbreaks of disease are dependent on these factors. During a two year study in East Baton Rouge Parish, La., greater than 97% of biting midges were collected from March to October when the mean daily temperature was between 10°C

and 33°C (Sabio 2000). Rainfall can also play an important role in biting midge populations by increasing the number of available sites for development (Gerry and Mullens 2000). Temperature can have an impact on the replication rate of BTV, generation time from egg to adult, and the survival of adult biting midges. At higher temperatures, infection rates and virogenesis is higher, but the life spans of flies become shorter (Mullens et al. 1995). Freezing temperatures kill adult midges which limits survival in winter (Mellor et al. 2000).

The seasonal distribution and abundance of *Culicoides* are very important to record when characterizing an epizootic of disease agents transmitted by biting midges (Mellor et al. 2000). The seasonality of disease outbreaks is correlated with the timing of annual peaks for adult specimens of competent vectors of *Culicoides* (Mohammed and Mellor 1990). Establishing the occurrence of adults of *Culicoides* species during the winter months also is important in understanding how viral agents can overwinter (Mellor 1996). Sellers and Mellor (1993) suggested that some viruses transmitted by specimens of *C. imicola* may overwinter in adult flies in locations with a daily maximum temperature of at least 12.5°C in the coldest month of the year. Bluetongue virus can sometimes be transmitted by insects which are not normally considered vectors. For example, Mellor and Boorman (1980) showed that *C. nubeculosus* Grogan and Phillips, which is not normally a vector for BTV, can be a vector for BTV when simultaneously infected with BTV and microfilariae of *Onchocerca cervicalis.*

Long range wind dispersal of adult biting midges has been documented and is considered to be important in the movement of arboviruses. The idea is that infected midges can survive transportation over long distances via large wind events and

subsequently transmit pathogens via the bite uninfected host in the new area. Mellor et al. (2000) referenced sixteen articles regarding long-range dispersal of *Culicoides* specimens on wind currents. Murray (1987) presented convincing data associating Akabane disease outbreak in New South Wales with evidence of long-distance dispersal of *Culicoides brevitarsis* Keifer the Hunter Valley. There was a severe drought in the area of the outbreak, and this area is also not included in the normal geographic distribution of *C. brevitarsis*. El Fatih et al. (1987) also demonstrated proof for spread of BTV associated with prevailing winds in the Sudan. Strong wind events, such as hurricanes, are common in south Louisiana and wind dispersal of exotic species of *Culicoides* has been suggested. Recently, in 2007, experts at USDA's National Veterinary Services Laboratories (NVSL) announced that BTV serotypes 3, 5, 6, 14, 19, and 22 were isolated and identified from Florida (SCDWDS 2008). These BTV serotypes had never been reported in the U.S., and could be here a result of long range wind dispersal of exotic competent vectors.

1.4 Bluetongue Virology, Overwintering and Disease

Bluetongue virus is composed of double-stranded ribonucleic acid (RNA) and placed in the genus Orbivirus, family Reoviridae. The genome is made up of 10 genes which encode messenger RNA's for seven structural and three nonstructural proteins. The RNA genome is encapsulated in a double-layered protein coat (Roy et al. 1990). Two major proteins, viral protein 2 (VP2) and VP5, are contained in the outer coat. The specificity and determination of serotypes resides in the VP2 gene (Mecham et al. 1996). Proteins VP3 and VP7 make up the inner coat and VP7 has been shown to be the protein involved in virus attachment (Xu et al. 1997). An infectious sub particle is produced

when VP2 is cleaved from the outer capsid and an inner core particle results from further enzyme treatment (Mertens et al. 1987).

Bluetongue virus is known to infect all species of ruminants, and the World Organization of Animal Health (OIE) maintains that BTV is a transmissible disease agent that has the potential for serious, rapid spread, and is of major importance in the international trade of animals and animal products. There are international regulations that prohibit the movement of livestock and relative products from BTV endemic areas to BTV-free areas, and these regulations create indirect losses for livestock producers (Blackwell, 2004). Tatem et al. (2003) indicated that the worldwide economic impact of BTV was in the order of 3 billion USD per year. Bluetongue virus has been isolated in bull semen, and heifers have been shown to contract BTV through insemination with BTV-infected sperm (Bowen 1983; Luedke et al. 1975). Therefore, there is also a restriction on the sale or trade of bull semen which can have a substantial impact on the cattle industry.

The overwintering mechanism of BTV is not well understood. In temperate regions with freezing winters, the *Culicoides* can survive as larvae and the transmission cycle is interrupted. In temperate regions without a true winter, BTVs may be maintained in year-round vector to host transmission cycles (Gerry & Mullens 2000). But even in these regions, the cycles may be interrupted by severe environmental conditions and clearly a mechanism is required to bridge BTV from one season to the next (Gibbs et al. 1992). Some studies have shown that BTVs are not transmitted transovarially in colonized *C. sonorensis* using virus isolation on vertebrate cells, but viral antigen was detected in ovarian and reproductive-tract tissues, which could indicate possible vertical transmission (Jones & Foster 1971; Nunamaker et al. 1990). White et al. (2005) found

BTV genome segments 7 and 3 using PCR in larvae of *C. crepuscularis* collected in cattle pastures in Colorado, but they did not detect all segments or culture BTV from larvae. This further indicates a possible vertical transmission in another species of *Culicoides*. More studies need to be carried out to determine if other species can transmit BTV from parent to offspring. The other proposed overwintering mechanism is in the ruminant host, but studies have not been able to clearly show BTV truly overwintering in the mammalian host and becoming infectious from one season to the next (Wilson et al. 2008).

Bluetongue disease (BT), which was first reported in South African sheep (Hutcheon 1902), can cause severe morbidity and mortality in sheep of certain breeds and deer of some species, especially Merino sheep and white-tailed deer (Mellor et al. 2000). Some common symptoms of bluetongue disease include fever, lameness, oral lesions, swollen muzzle, necrosis of the tongue, and hemorrhaging of the coronary bands of infected adults. Lack of oxygen to the tongue, which is often observed in infected sheep, can cause swelling and a blueish color, hence the name "blue tongue". Less than 5% of infected adult cattle show any clinical signs, but cattle can develop a prolonged viremia lasting several weeks, which makes cattle ideal reservoir hosts for BTV. Early prenatal BTV infection can lead to embryonic death resulting in abortions or still births in cattle (Tabachnick 1996).

1.5 BTV Serotype Distribution, Vectors of BTV, and Vector Competence

Currently, there are 26 different recognized serotypes of bluetongue virus (genus *Orbivirus*; family Reoviridae) distributed differentially worldwide (Maan et al. 2012). In the Central American-Caribbean Basin, BTV serotypes 1, 3, 4, 6, 8, 12, 14, and 17 have been observed (Tanya et al. 1992; Thompson et al. 1992). In Australia, where BTV

serotypes 1, 3, 9, 15, 16, 20, 21, and 23 are transmitted by *C. wudui, C. breviturs*is, *C. fulvus, and C. ucfoni***,** there is not much clinical disease in ruminants (Tabachnick 1996). Serotypes 1, 2, 3, 9, 12, 14, 19, 20, 21, and 23 occur in Asia where several species of *Culicoides* are known to be vectors (Taylor 1986). Serotypes 1-19, 22, and 24 are found in Africa and the Middle East, where the primary vector is C. *imicola* (Mellor 1990). In many parts of the world, BTV vectors are unknown due to lack of research on the subject (Tabachnick et al. 1992).

Recently BTV-8 has caused a severe epizootic of bluetongue disease (BT) in northern Europe. Prior to 1998, BT was considered to be an exotic disease in Europe. From 1998 through 2005, 5 serotypes of BTV (1, 2, 4, 9, and 16) were detected in the Mediterranean Basin (Saegerman et al. 2008). The suspected BTV vector in Europe was *C. imicola* (primary BTV vector in Asia and Africa), which has now been recorded as far north as 44°N (Goffredo et al. 2001). However, in the region of the outbreak in 2006, a pool of 50 non-engorged, parous *C. dewulfi* (Goetghebuer) collected in the Netherlands were PCR positive for BTV (Meiswinkel 2007). Also, Savini et al. (2004) isolated BTV from field collected specimens of the *C. obsoletus* complex in central Italy. Furthermore, BTV was isolated from field-collected specimens of *C. pulicaris* in Sicily (Caracappa et al. 2003)**.** Therefore, it has been suggested that several species of *Culicoides* in Europe can be competent vectors of BTV.

Until the discovery of BTV-1 in south Louisiana in 2004, 5 of the 24 serotypes of BTV were known to occur in the U.S. (Mullen et al. 1999); serotypes 2, 13, and 17 were known to occur in Louisiana (Wieser-Schimpf et al. 1993). Since 1998, an additional 10 serotypes (1, 3, 5, 6, 9, 12, 14, 19, 22 and 24) have been reported to occur in the

southeastern United States from cattle and deer (Maclachlan et al. 2013). BTV-1 was first reported in the U.S. by Johnson et al. (2006) in south Louisiana where it was isolated from a hunter-killed white-tailed deer and serotypes 3, 5, 6, 9, 14, 19, 22 and 24 were isolated in 2007-2008 from deer and cattle in Florida (Ostlund 2010). BTV-12 was first reported in Texas from deer tissue in 2008 (Stallknecht and Fischer 2009). Since 2006 BTV-3 has been isolated in Mississippi, Arkansas, South Dakota and most recently in Texas (Ostlund 2006). BTV-3 was first detected in Florida in 1999 and was isolated for the next several years.

The global distribution of bluetongue viruses corresponds to the distribution of *Culicoides* vectors (St. George and Kegao 1996). The only proven competent vectors of BTV are members of the genus *Culicoides* (Kramer et al. 1985; Ward 1996; Ward 1994; Hoar et al. 2004). Worldwide, at least seven species of *Culicoides* are considered major vectors of BTV, although numerous species are considered possible vectors (Paweska et al. 2002; Tabachnick 2004). In the Central American-Caribbean Basin, the most likely BTV vector is *C. insignis* (Tanya et al. 1992). In Australia, BTV is transmitted by biting midges of *C. wudui, C. brevitursis, C. fulvus,* and *C. ucfoni* (Tabachnick 1996), and in Africa and the Middle East the primary vector is *C. imicola* (Mellor 1990). In Asia, the species of *Culicoides* that transmit BTV are unknown (Mellor et al. 2000). In the United States, species of the *Culicoides variipennis* complex and *C. insignis* are the only proven vectors of BTV (Tabachnick, 1996, Tanya et al.1992). Until Holbrook et al. (2000) clarified that what was thought to be one species (*C. variipennis*) was actually three species (*C. sonorensis, C. occidentalis, and C. variipennis*); it was believed that there were geographic populations of *C. variipennis* that were refractory to BTV infection. Specimens of *C. insignis* are considered the BTV vector in Florida because they have

been shown to transmit the virus in laboratory settings and field caught specimens were PCR positive for BTV (Greiner et al. 1985).

In the U.S., bluetongue virus was first isolated from sheep in California in 1952 (McKercher et al. 1953). Foster and Jones (1963) demonstrated that *C. variipennis* was a biological vector of BTV after feeding specimens on infected sheep, incubating the specimens for 10-15 days, and then allowing them to feed on non-infected sheep who then became infected. The authors also isolated BTV using cell culture from specimens of *C. variipennis*. Jochim et al. (1966) first showed that BTV replicated in specimens of *C. variipennis* that were intrathoracically inoculated with BTV, and Bowne and Jones (1966) showed that BTV replicated in the salivary glands of *C. variipennis*. Kramer et al. (1990) isolated BTV-11 using cell culture techniques from field-caught *C. variipennis* in Colorado and Utah. Wieser-Schimpf et al. (1993) reported BTV serotypes 2, 13, and 17 from seropositive cattle in Baton Rouge, Louisiana, and found 1 out of 135 pools of *C. variipennis* to be positive for BTV via PCR, but the serotype was not reported.

Currently, *C. sonorensis*, which occurs in the south and southwest U.S., is considered to be the principal vector of BTV in the United States because of vector competence and field studies (Holbrook and Tabachnick, 1995; Tabachnick, 1996; Tabachnick, 2004). The other two species from the complex (which are refractory for BTV) are *C. variipennis*, which occurs mostly in the East and South and *C. occidentalis*, which occurs in the Southwest (Holbrook et al, 2000; Tabachnick, 1996). Therefore, it should be noted that in discussions of references to work conducted before the publication of Holbrook et al. (2000) that *C. variipennis* is a likely synonym for C. *sonorensis* for vector competence studies. Specimens of *C. variipennis* are not considered BTV vectors because they have a low susceptibility rate to infection in the lab, and no

virus has been isolated in field-collected specimens (Tabachnick 1996). Specimens of *C. occidentalis* collected at Borax Lake in California also had low susceptibility rates for BTV and no virus has been isolated from midges of this species (Tabachnick 1996).

The epidemiology of BTV transmission in the U.S. with *C. sonorensis* as the primary vector has been generally accepted (Holbrook and Tabachnick, 1995; Tabachnick, 1996; Tabachnick, 2004). In Louisiana, an association of transmission of BTV-13 and BTV-17 with seasonal peaks of *C. variipennis* was shown, and also BTV RNA was found in one of 381 pools (6,072 flies) of *C. variipennis* via PCR (Wieser-Schimpf et al. 1993). Also, entomological studies on BTV/EHDV in Louisiana have implicated 4 species to be probable vectors including *C. crepuscularis, C. debilipalpis, C. stellifer,* and *C. haematopotus* after BTV positive pools were determined from specimens captured in light traps at locations where BTV was being transmitted among cattle or deer (Becker et al. 2008, Becker et al. 2020)

1.6 Epizootic Hemorrhagic Disease Virus and Disease

Epizootic hemorrhagic disease virus (EHDV) is made up of double stranded RNA *orbivirus* composed of 10 double stranded (ds) RNA segments (Huismans et al. 1979; Mecham and Dean 1988). The genome codes for seven structural proteins and three non-structural proteins. Genome segment 2 codes for the major viral protein and determines serotype specificity and induction of neutralizing antibody (Mecham and Dean 1988). Genome segments 1, 3, 4, 6, and 8 are highly conserved with more than 90% homology among cognate genes of other EHDV serogroups (Wilson et al. 1990). Shope et al. (1960) first isolated EHDV serotype 1 after an epizootic of hemorrhagic disease causing a large white-tailed deer die off in 1955 in New Jersey.

Epizootic hemorrhagic disease virus (EHDV) is almost identical to BTV morphologically, however they differ antigenically. Another difference between the two viruses is that BTV causes severe disease in sheep, but EHDV does not affect sheep (Fletch and Karstad 1971). The clinical disease and symptoms in white-tailed deer are identical for BTV and EHDV, and only virus isolations or the use of PCR can differentiate between the two viruses. "Hemorrhagic disease" is the collective term often used to describe disease in wild ruminants caused by BTV or EHDV. In domestic ruminants, BTV causes bluetongue disease (BT) and EHDV causes epizootic hemorrhagic disease (EHD). Deer with epizootic hemorrhagic or bluetongue disease can develop severe internal hemorrhaging in major organs such as the spleen and liver which leads to death. Clinical signs of epizootic hemorrhagic or bluetongue disease include anorexia, weakness, nasal mucosa, salivation, and sometimes necrosis of the tongue or on the coronary bands (Fay et al. 1956). The pathogenesis of epizootic hemorrhagic or bluetongue disease directly results from vascular endothelial cell damage due to viral replication and rupture of these cells (Tsai and Karstad 1971).

Recently, Fox et al. (2015) suggested an association between EHDV infection vascular and inflammatory lesions of the testis and epididymis and cactus buck antler abnormalities seen in Colorado mule deer. One deer in the study was PCR positive for EHDV in the testis but not in the spleen, indicating that EHDV may exhibit a targeted infection, which is unexpected because it is commonly found in the spleen of ruminants if there is any infection at all. In another study, EHDV-6 was used to inoculate 18 month old Holstein heifers to observe clinical signs and pathogenicity because they have been observed in the field in cattle in certain outbreaks (Breard et al. 2013). Although viremias spiked after exposure, very mild clinical signs were observed. The authors

suggested that the age of the cattle may predict clinical signs and older cattle should be used for future experiments. Similarly, Ruder et al. (2012) inoculated a group of white-tailed deer with EHDV-7 and all of them became infected with different degrees of clinical symptoms. They observed a 67% mortality rate and only one deer that was mildly affected, but all animals developed detectable viremia and seroconverted during the study. This study shows that white-tailed deer can be a highly susceptible ruminant hosts for an exotic serotype of EHDV in North America.

1.7 EHDV Serotype Distribution, Vectors, and Vector Competence

At least 7 serotypes of EHDV are distributed worldwide (Gorman 1992). Currently, little is known about the global epidemiology of EHDV (Aradaib 2004). In Nigeria, serotypes 3 and 4 were isolated from *Culicoides spp* (Moore 1974). In Australia, 6 serotypes have been identified (Weir et al. 1997). Ibaraki virus, which has been identified as EHDV-2, caused an extensive outbreak in cattle in Japan in 1959 and continues to sporadically cause disease outbreaks (Inaba 1975).

In the U.S., EHDV serotypes 1, 2, and 6 have been reported. Up until 2006, EHDV-1 had been isolated from Mississippi and Missouri and EHDV-2 had been isolated from deer from Colorado, Georgia, Illinois, Kansas, Louisiana, Missouri, and Texas (SCWDS 2006). EHDV- 1 was first isolated and described by Shope et al. (1955) after an epizootic of hemorrhagic disease in white-tailed deer in New Jersey and EHDV-2 was described by Foster et al. (1977) in Kentucky. Since then, both EHDV-1 and EHDV-2 have been considered endemic in much of the U.S. and have caused sporadic disease outbreaks in white-tailed deer populations. The first isolation of EHDV-6 came from sick or dead white-tailed deer in Indiana and Illinois. Genetic studies indicated this was a reassortant of an exotic EHDV-6 and a native EHDV-2. The data was unclear as to

the origins of the exotic parental EHDV-6, but recently in 2010 it was detected in Guadeloupe and is circulating in the Caribbean (Allison et al. 2012). The reassortment took place in the U.S., and this was the first report of a field isolation of an EHDV reassortant of exotic and endemic serotypes (Allison et al. 2010). Since 2006, EHDV-6 (Indiana) has been isolated in 12 states and appears to be becoming endemic to U.S. In 2012, cattle samples were found PCR positive for EHDV-6 and EHDV-2 at the Bob R. Jones Idlewild Research Station near Clinton, La (Becker et al. 2020).

The only known vectors of EHDV are biting midges in the genus *Culicoides*. In Africa, EHDV has been isolated from midges in the *C. schultzei* group and in Australia the virus has been isolated in *C. brevitarsis* (Parsonson and Snowdon 1985). In Sudan, the primary vector of EHDV is *C. imicola* (Aradaib 1999), but the vectors of EHDV are unknown in Central America, South America, Japan, and Southeast Asia (Mellor et al. 2000).

In the U.S., the primary vector of EHDV is considered to be *C. sonorensis.* Foster et al. (1980) reported that both strains of EHDV (1 and 2) were isolated from cattle and wild-caught specimens of *C. sonorensis*. More recently, Smith et al. (1996) isolated EHDV-2 from specimens of both *C. debilipalpis* and *C. variipennis* that fed on viremic deer and tested up to 15 days later. In Arizona, Rosenstock et al. (2003) tested field captured specimens of *C. mohave* for EHDV, and reported that 35% of the pools were PCR positive. Moreover, Ruder et al. (2012) demonstrated that specimens of *C. sonorensis* were able to transmit EHDV-7 to naïve white-tailed deer by allowing the infected midges to feed on the deer which subsequently developed severe clinical disease. Recently, McGregor et al. (2019) reported EHDV positive pools from field collected specimens of *C. stellifer* and *C. venustus* in Florida at a location with apparent EHDV

transmission. In the U.S., although *C. sonorensis* is the primary vector for EHDV among cattle and sheep, it is largely unknown what vector is responsible for EHDV transmission among white-tailed deer, especially in the wild. In fact, many studies have shown an absence of *C. sonorensis* in areas of apparent EHDV transmission among deer.

1.8 Management and Control of *Culicoides*

Hemorrhagic disease, caused by epizootic hemorrhagic disease virus (EHDV) or bluetongue virus (BTV), is the most important viral disease of white-tailed deer. The accepted vectors of these viruses are members of the genus *Culicoides*. There are over 1,400 species of *Culicoides* and female specimens of 96% are obligate blood-feeders that feed on mammals and birds (Meiswinkel et al. 1994). Many species of *Culicoides* have unique habitats and ecological niches of which many are unknown and understudied. Methods for controlling biting midges in the genus *Culicoides* have proven to be quite difficult due to the lack of knowledge on basic biology and behavior of these tiny insects, especially in areas where there is an outbreak of hemorrhagic disease in white-tailed deer.

BTV and EHDV are both arboviruses, or arthropod-borne, meaning they need an insect or arthropod to transmit the virus among susceptible hosts. Competent vectors in the genus *Culicoides* are required to transmit these viruses among susceptible ruminant hosts. In fact, transmission models of vector- borne diseases show that the most important factor of disease transmission is the lifespan of the adult insect (MacDonald 1957). Therefore, many control measures have been directly associated with interrupting or killing the adult midges that vector the virus. Currently, the most common method to control midges is the application of insecticides, usually pyrethroids, directly onto livestock because such little is known about larval habitats and adult resting places of

midges. Ivermectin is effective at killing both adults and larvae of *Culicoides* species but also can be harmful to beneficial insects (Webb et al. 2010).

Recently in the U.S., white-tailed deer farming is a growing business contributing almost 3 billion U.S. dollars to the economy and 29,000 jobs in 2007(Anderson et al. 2007). These farms often experience severe epizootics of hemorrhagic disease that lead to high mortalities which can cause significant economic consequences from lost revenue. Therefore, it has become highly relevant and important to understand how to reduce hemorrhagic disease and mortality in farmed white-tailed deer targeted toward controlling the biological vectors, biting midges in the genus *Culicoides*.

Ansari et al. 2011 showed that entomopathogenic fungi may be a potential biocontrol method for targeting adult biting midges; they found that *Metarhizium anisopliae* was the most virulent causing a significant decrease in midge survival compared to all other strains tested. However, this method has not been tested on a large-scale field situation to evaluate its efficacy. Laboratory trials using a wind tunnel to expose adult *Culicoides* to insecticides showed that synthetic pyrethroids caused better kill rates than organophosphates, but the results were highly variable (Kline et.al. 1981; Floore 1985). One study found no reduction in the number of adult *Culicoides* after spraying a micro-encapsulated formulation (including cypermethrin, piperonyl butoxide, and pyrethrin synergists) from a vehicle mounted vaporizer (Satta et al. 2004). Using insecticide treated netting as a barrier is another method which has been examined for controlling adult midges around livestock (Schreck and Kilne 1983). Kline and Roberts (1981) showed that an 8% formulation of propoxur on an aluminum mesh resulted in mortality of over 97% for 35 days after treatment. Mullens et al. (2001) found that

applying 0.2% w/v permethrin to the ventral portion of 200 cattle had no significant effect on BTV transmission.

The majority of trials to test different larvicides against midges in the genus *Culicoides* have concentrated on species which are nuisance pest of man rather than those of veterinary importance. Holbrook and Agun (1984) reported high mortality rates in *Culicoides sonorensis* larvae in a field application using granular organophosphates and pyrethroid sprays near pond margins. This is important information for studying hemorrhagic disease transmission in white-tail deer because *C. sonorensis* is the primary vector of BTV in the U.S. Another method to control biting midges is protective stabling of livestock. The problem associated with this control method is finding midge-proof housing and determining if the local species of *Culicoides* is endophilic or exophilic. Lastly, the disruption of breeding sites and habitat modifications has been shown to decrease populations of midges. Good management practices, such as fixing overflowing water troughs or leaky pipes, can prevent the creation of conducive environments capable of producing midge larval habitats (Mellor and Wittmann 2002). The larval habitat of the main BTV vector in the U.S., *C. sonorensis*, is well defined and breeding sites are characterized including such parameters as soil chemistry (Schmidtmann et al. 2000), pollution levels (Mullens and Rodriguez 1988) and shading (Mullens and Rodriguez 1985). By eliminating one or more of these components, it may be possible to control local populations of *C. sonorensis* by removing or interrupting an essential component to the larvae life stages. Mullens (1992) suggested that reducing water levels near cattle could lower midge populations significantly.

In the United States, species of the *Culicoides variipennis* complex and *C. insignis* are the only proven vectors of BTV (Tabachnick, 1996). Until Holbrook et al.

(2000) clarified that what we thought was one species (*C. variipennis*) was actually three species (*C. sonorensis, C. occidentalis, and C. variipennis*), it was believed that there were geographic populations of *C. variipennis* that were refractory to BTV infection. The main vector of BTV is *C. sonorensis* and determining if this species is present in an area can play a major factor in studying BTV and EHDV transmission, especially on deer farms. However, other species of *Culicoides* such as *C. debilipalpis, C. crepuscularis, C. venustus, C. stellifer* and *C. venustus* may be important vectors of BTV and EHDV in areas BTV/EHDV transmission is apparent but *C. sonorensis* is absent (Becker et al. 2010, Becker et al. 2020, McGregor et al. 2019).

1.9 Novel Viruses transmitted by *Culicoides*

In 2011, an unidentified disease which caused clinical signs such as fever, decreased milk production and diarrhea was reported for cattle in Germany, the Netherlands and Belgium (Hoffmann et al. 2012). In November 2011, genomic sequences of a new virus were detected by the Friedrich-Loeffler-Institut, Germany, in a pool of cattle blood samples and was named Schmallenberg virus (SBV) (Hoffmann et al. 2012). Phylogenetic analysis showed that SBV belongs to the Simbu serogroup within the Orthobunyavirus genus and the Bunyaviridae family, and this was the first report of a virus from this serogroup in Europe. In the 2 years following its identification, SBV spread rapidly causing malformations and stillbirths in cattle, goats and sheep to many surrounding countries in Europe (Afonso et al. 2014). The only proven vector established under laboratory conditions is for *C. scoticus* (Veronesi et al. 2013), but field data have confirmed the vector competence of midges of the *C. obsoletus* complex, *C. dewulfi* and *C. chiopterus* (De Regge et al. 2012).

In 2008 Toggenburg Orbivirus (TOV), a new BTV serotype classified as BTV-25 was detected in goats in the Toggenburg region of Switzerland (Hoffmann et al. 2008). The biological vector of BTV-25 is unknown and preliminary studies showed this virus may be transmitted trans placentally in goats. In early 2010, a novel strain of BTV was detected in a sheep and goats in Kuwait and the virus was characterized as bluetongue virus serotype 26 (Maan et al. 2001). It was discovered that BTV-26 replicates to high levels in goats, suggesting that goats may be the natural host for this virus and BTV-26 may be spread by direct contact transmission with no biological vectors required (Batten et al. 2013). Neither BTV-25 or BTV-26 cause clinical symptoms in goats but mild symptoms can occur in sheep such as fever, decreased appetite, and facial discharge (Batten et al. 2012).

In 2006, samples were taken from a farmed 18 month old white-tailed deer exhibiting clinical signs of epizootic hemorrhagic disease and submitted to University of Illinois Veterinary Diagnostic Laboratory (Cooper 2014). The unknown orbivirus virus was tentatively named mobuck virus because it was isolated from a buck raised in Missouri. This virus was also isolated in Florida in deer (Ahasan et. al 2019). Very little is known about this virus and the transmission cycle but it is very closely genetically related to EHDV. Recently, Ahasan et al. (2020) reported 3 new orbiviruses found in white-tailed deer from Florida. Phylogenetic analyses indicate that these new orbiviruses are genetically closely related to the Guangxi, Mobuck, Peruvian horse sickness, and Yunnan orbiviruses and 4 of the 6 deer samples analyzed were co-infected with EHDV-2.

CHAPTER 2. COMPARING DIFFERENT TRAP TYPES FOR COLLECTING CULICOIDES SPECIMENS INFECTED WITH BTV OR EHDV

2.1 Introduction

Over 50 different arboviruses have been isolated from ceratopogonid midges of the genus *Culicoides* worldwide (Mellor et al. 2000). The most important human pathogen transmitted by *Culicoides* midges is Oropouche virus (OROV), a member of the Orthobunyavirus genus in the Bunyaviridae family, which causes Oropouche fever. This virus is endemic in parts of Central and South America and causes Dengue-like symptoms including fever, chills, and headaches. The principal vector for Oropouche virus is considered to be *C. paraensis* (Pinheiro et al. 1976, Pinheiro et al. 1981). African horse sickness (AHS) virus is a double stranded RNA Orbivirus in the family Reoviridae, which is primarily transmitted by *C. imicola* in Africa, and can cause mortality rates in excess of 95% in equid populations (Mellor 1990). Outbreaks of AHS have been reported from South Africa to as far north as Spain and Portugal. More recently, the *Culicoides*-borne Schmallenburg virus (family Bunyaviridae in the Simbu serogroup) which causes birthing defects of ruminants has been described in northern Europe, and multiple species of *Culicoides* including C. *scoticus, C. obsoletus sensu stricto,* and *C. chiopterus* are considered to be biological vectors of this virus (Elbers et al. 2013).

Bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV) are in the genus *Orbivirus,* family Reoviridae and are transmitted to ruminants by infected *Culicoides* midges (Tabachnick 1996). These viruses are distributed worldwide and the occurrence of these 2 viruses is described by the detection of antibodies or clinical disease in ruminants which are all susceptible to BTV and EHDV infections. In cattle, infections of BTV and EHDV are normally subclinical with long lasting viremia.

Therefore, cattle are considered reservoirs of these viruses. In domestic ruminants BTV causes bluetongue disease (BT) and EHDV causes epizootic hemorrhagic disease (EHD). Hemorrhagic disease (HD) in wild ruminants is caused by the infection of BTV or EHDV, but the highest mortality and morbidity is observed in white-tailed deer and sheep (Tabachnick 1996). There are 26 recognized serotypes of BTV and 7 EHDV recognized serotypes distributed worldwide that are associated with different vector species of *Culcoides* (Maan et al. 2012).

Five bluetongue virus serotypes (2, 10, 11, 13, and 17) were known to be present in the U.S. before 1998; an additional 10 serotypes (1, 3, 5, 6, 9, 12, 14, 19, 22 and 24) have since been reported from cattle and deer (Maclachlan et al. 2013). For EHDV, serotypes 1, 2 and 6 are the only three reported in the U.S.(Ruder et al. 2015). In the U.S. the only two confirmed vectors of BTV/EHDV that have been shown to be infected after feeding on susceptible hosts and subsequently transmit the virus to a susceptible host are *C. sonorensis* Wirth and Jones and *C. insignis* Lutz (Maclachlan et al. 2015). The species *C. sonorensis* is one of a complex of 3 species along with *C. occidentalis* and *C. variipennis.* Holbrook et al. (2000) separated *C. variipennis* into these 3 species; therefore in any literature prior to 2000, *C. sonorensis* is synonymous with *C. variipennis* but not necessarily vice versa.

There have been numerous field studies with reported transmission of BTV and EHDV when *C. sonorensis* was rare or absent. In one study, only 1 specimen of *C. variipennis* was collected during a study using light traps and direct aspirations from white-tailed deer during an epizootic of hemorrhagic disease; the majority of specimens were from *C. debilipalpis*, *C. paraensis*, and *C. stellifer* (Smith and Stallknecht 1996). Similarly, Smith et al. (1996) collected over 200,000 specimens of *Culcioides* from

white-tailed deer in Georgia at a site enzootic for hemorrhagic disease including specimens of *C. debilipalpis, C. stellifer, C. biguttatus, C. venustus,* and *C. arboricola* but did not capture a single specimen of *C. variipennis*. Furthermore, Becker et al. (2010) collected specimens from 10 species of *Culicoides* in Louisiana over a 2 year period in an area with BTV transmission of which 4 pools of flies (*C. crepuscularis, C. debilipalpis, C. haematopotus,* and *C. furens*) were PCR positive for BTV; no specimens of *C. sonorensis* were captured throughout the study. More recently, McGregor et al. (2018) have implicated *C. venustus* and *C. stellifer* as vectors for EHDV after detecting positive pools from field-collected specimens during an epizootic of EHD at white-tailed deer farms in Florida where *C. sonorensis* was absent. Therefore, it is important to determine the local species of *Culicoides* in areas with BTV/EHDV transmission. The majority of the studies referred to above used light traps to collect *Culicoides* midges in an area of known orbivirus transmission.

There have been many studies to determine the most efficient light trap type for capturing specimens of *Culicoides*. Rowley and Jorgensen (1967) caught almost 11 times more specimens of *Culicoides* spp. in New Jersey traps modified with black light compared to the standard incandescent light. Holbrook (1985) found that the addition of dry ice as bait to black light miniature CDC traps (Model 512, John W. Hock Co., Gainesville, FL 32604) increased capture of C. *variipennis* 17 times more than traps without dry ice. In Louisiana, Becker (2008) showed that miniature CDC black light traps captured almost 5-fold as many specimens of *Culicoides* than did combined New Jersey traps with incandescent and New Jersey traps with black light.

Although light traps are the gold standard for catching specimens of *Culicoides* in the field, there are inadequacies of using these traps as a sole technique in studies on

virus transmission. Inherently, light traps catches will differ on individual trap nights depending on light intensity and abiotic factors such as weather conditions or moon phase and do not necessarily capture all of the species in natural proportions. Certain species of *Culicoides* are diurnal and can only be captured during the daylight hours; for example, female specimens of *C. paraensis* have been reported biting humans during daylight hours (Pinheiro et al. 1981). Therefore other traps, such as animal baited traps or vehicle mounted traps, have also been utilized to capture specimens of *Culicoides*. Barnard (1980) concluded that airborne populations of *C. variipennis* are not well represented by light trap catches during the full moon and collected significantly more specimens using a vehicle mounted trap. In one study, Viennet et al. (2011) used animal baited traps versus UV-light traps and found 2 species of *Culicoides* in the animal baited traps that were not present in the light traps. In addition, Carpenter et al. (2008) found that *C. chiopterus* was substantially underestimated using light traps versus drop traps using live sheep; prior to that study, *C. chiopterus* was not considered to be a BTV vector due to the low numbers of specimens previously captured in light traps. The authors stated the reason they did not catch *C. chiopterus* in light traps is unknown but not due to the fact that it is a diurnal species because activity levels under the conditions examined were similar to other species. There have been studies to show that the physiological status of *C. sonorensis* can affect trap catches. Wieser-Schimpf et al. (1991) found that a black light trap with CO_2 collected significantly more nulliparous and parous empty *C. variipennis* than a black light trap without CO_2. Mayo et al. (2012) collected *C. sonorensis* midges using CDC traps with and without black light and directly from cattle and found higher infection rates in *C. sonorensis* midges collected without light and directly from cattle. Subsequently, McDermott et al. (2015) used suction traps with and without light in an

area of BTV transmission and captured significantly more BTV infected *C. sonorensis* in traps with no light, and attributed the results to BTV infecting the eyes of the midges thus negating the effects of light. Although the importance of using more than one trapping method when assessing risk for BTV transmission by *C sonorensis* is recognized, few studies on this subject have been conducted on other potential vectors of BTV and EHDV in the U.S..

This study was conducted to address the needs of identification of trapping methods that are adequate for collecting vectors of orbiviruses in the southeast U.S. in areas without intensive animal agriculture such as dairies. The first objective of this study was to compare the number of species, abundance, and virus infection rate of female *Culicoides* collected in dry ice baited CDC traps either with or without black light to test the hypothesis that infected midges are repelled by light. The second objective was to compare the number of captured midges of *Culicoides* and species present at different crepuscular and nocturnal periods using a trap that collected insects in 2 hour time intervals from 18:00h to 08:00 h. The final objective of this study was to determine which species of *Culicoides* were present in an area of BTV and EHDV transmission among captive ruminants using 5 different trap types: animal baited drop trap, animal baited baffle trap, direct animal aspirations, CDC trap with black light, and CDC trap with no light.

2.2 Materials and Methods

The study was conducted at the Louisiana State University Agricultural Center Bob R. Jones Idlewild Research Station (BJIRS) near Clinton, Louisiana (30.817954N, 90.97324W) from August through September of 2014 in an area near a forest edge in the proximity of deer pens and cattle pastures. The station maintains a reproductive herd of

approximately 100 captive white-tailed deer as well as reproductive herds of around 50 crossbred beef cattle and 30 red deer (*Cervus elaphus*).

- Light vs No Light Trap Study

From January 2016 through December 2018, six miniature CDC black light traps (model 512; John W. Hock Co., Gainesville, FL) baited with 2 kg of dry ice in igloo containers were deployed before dusk and collected the next morning after sunrise. The traps were hung approximately 1.5 m above ground at 3 different locations. The first location was along the fence line outside and adjacent to a white-tailed deer pen where approximately 50 deer were present year round. The second location was along the fence of a cattle pasture where 35 cows were present. The third location was in a forested area with a mixture of hardwood and pine trees approximately 5 km away from the nearest cattle pasture or deer pen. Each location had 2 trap sites greater than 50m apart receiving one of the 2 treatments alternately, and there was a total of 240 trap nights from 2016 to 2018. The light was removed from one of the two traps at each location and the treatment was rotated for each trapping night so that the trap with light became the trap without light on the next trapping occasion. After collection the insects were held at a 4°C until the specimens were sorted using a dissecting microscope and a chill table (BioQuip®, Gardena, CA). Members of the genus *Culicoides* were sorted by species through examination of wing patterns using the keys of Blanton and Wirth (1979); voucher specimens were confirmed by mounting, dissecting and clearing followed by examination of the spermathecae.

Analysis of variance (ANOVA) tests was performed on the mean number of specimens captured for the light versus no light study. Minimum infection rates (MIR)

were calculated by number of positive pools divided by number of specimens tested multiplied by 1000 (Luedke et al. 1977).

The collected midges from the genus *Culicoides* were pooled by trap type, species, site, and date and placed into Eppendorf 2.0mL Safe-Lock tubes (Cat. No 022363352). Pools of 5-50 midges were homogenized using a plastic pestle and a Kimble pellet pestle motor (model #749540). Then 900 uL BA-1 buffer consisting of Hanks M-199 salts, 0.05 M Tris, pH 7.6, 1% bovine serum albumin, 0.35g/L sodium bicarbonate, 100 U/mL penicillin, 100ug/mL streptomycin, 1ug/mL Fungizone was added to each vial. Viral RNA was isolated from the midge homogenate using the Qiagen QIAmp viral RNA mini kit (catalog number 52906; Qiagen, Germantown, MD) using the manufacturer's instructions. BTV and EHDV multiplex reverse transcription and real-time PCR (RT-qPCR) utilized the Qiagen Rotor-Gene Multiplex RT-PCR kit (catalog number 204972; Qiagen, Germantown, MD) kit. The methods described in Becker et al. (2020) were used to conduct RT-qPCR on the samples to detect for viral RNA from BTV and EHDV. Samples were considered EHDV positive when Cq values were less than 40 cycles and BTV positive when Cq values were 36 cycles or less.

- Rotator Trap Study

A collection bottle rotator (model 1512; John W. Hock Co., Gainesville, FL) was used with a CDC miniature black light trap baited with 2kg of dry ice as described above. Collection bottles containing 50 mL of 95% ethanol were used to collect insects. The trap was set to collect at the intervals 1800-2000h, 2000-2200h, 2200-2400h, 0000-0200h, 0200-0400h, 0400-0600h, 0600-0800h. The traps were deployed in July, August, or September over a three year period for a total of 15 collection nights; the rotator trap was deployed at the BJIRS along the fence line outside cattle pastures and white-tailed

deer pens. Specimens of Culicoides were sorted into species following the procedures described above.

- Trap Comparison Study

There were a total of 5 trap sites greater than 50 m apart each with a different trap type each night. The first 3 types of traps used stanchioned beef calves that were approximately 6 months old and weighed approximately 100 kg. The first trap type was an animal-baited baffle trap described in Foil et al. (1984) which was constructed by a wooden frame (1.83m x 1.83m x 1.83m) covered by 52 x 52 saran mesh cloth. Three baffles were constructed on the sides which allowed insects to enter and feed on a live calf which was stanchioned inside the trap from 45 minutes before dusk until 30 minutes after dark (approximately 1.5 hours). Specimens of *Culicoides* were collected using a mouth aspirator with HEPA filter (model 612; John W. Hock Co., Gainesville, Fl) the following morning, placed in a cooler with blue ice, transported to the lab, and stored at 4°C. The 2nd trap type was a modified version of the animal baited drop trap described in Carpenter et al. (2008). A metal frame approximately 2m x 5m x 2m was constructed to fit around the stanchion and hold up the fine mesh netting which was dropped around the animal at dark and the calf was removed. A calf was stanchioned 45 min before dusk and insects were allowed to fly in and feed on the calf. Insects were aspirated the next morning, as previously described, from inside the net and stored at 4°C. The 3rd trapping method was directly aspirating specimens of *Culicoides* off of a stanchioned calf 45 minutes prior to sunset using the InsectaZooka field aspirator (model 2888A; BioQuip Products, Rancho Dominguez, CA) with 40 x 40 mesh aluminum collecting cups (model 2888CB; BioQuip Products, Rancho Dominguez, CA) . Collections were made 3 times for 10 minutes each by continuously and thoroughly sweeping the aspirator along the

dorsal and ventral side of the calf, neck and head area, and each leg. The collecting cups were transported to the lab and stored at 4°C. The 4th trap type was a 6-volt Center for Disease Control (CDC) miniature black light trap (model 512; John W. Hock Co., Gainesville, Fl) baited with 2 kg of dry ice and hung on a metal pole approximately 1.5 meters above the ground. No-see-um collection bags with 0.45kg collecting cup (model 2801B; BioQuip Products, Rancho Dominguez, CA) were used to collect insects. The 5th trap was identical to the 4th trap with 2kg of dry ice as bait but with the black light bulb removed. The 2 CDC miniature black light traps were set out approximately 45 minutes before dusk and collected the next morning within 60 minutes after dawn, approximately 11 hours later. Collection bags were placed in a cooler with blue ice and transported to the lab and stored at 4°C.

The 5 trap types were selected at random and rotated in a Latin Square design until two full rotations were completed. All midges of *Culicoides* collected were sorted into species as described above. The mean number of specimens per trap-night for each of the 5 trap types was compared using one-way ANOVA and Tukey's test for separation of means (SAS Institute 2000).

2.3 Results

- Light vs No Light Trap Study

The CDC trap with black light baited with dry ice captured specimens from 11 species of *Culicoides*: *C. arboricola, C. biguttatus, C. crepuscularis, C. debilipalpis, C. haematopotus, C. furens, C. stellifer, C. neopulicaris, C. variipennis, C. villosopennis,* and *C. venustus*. The CDC trap without light captured midges from 8 of the same 11 species excluding *C. furens, C. neopulicaris,* and *C. villosopennis* (Table 2.1).

During the study, specimens from 5 *Culicoides* species (*C. crepuscularis, C. debilipalpis, C. haematopotus, C. stellifer,* and *C. venustus*) were found RT-qPCR positive for BTV or EHDV; and therefore, the numbers of specimens from these 5 species were used for analysis (Table 2.2). For the data combined for all 3 years (2016-2018), there was a significant difference (p=0.014) using one –way ANOVA tests in the total number of specimens captured in CDC traps with UV black light versus CDC traps without light. The mean number of specimens ± standard error (SE) for the CDC traps with UV black light was 17.1 ± 4.51 and the mean for the CDC traps without light was 6.8 ± 1.54. The data were also analyzed by year using one-way ANOVA test; for 2016 there was no significant difference (p=0.510) in total number of specimens captured for CDC traps with and without black light. However in 2017 (p=0.029) and 2018 (p=0.037), there was a significant difference in the mean number of specimens captured in both years. The mean number of *Culicoides* specimens captured in CDC traps with black light was 26.4 ± 4.81 and without light was 7.1 ± 1.34 in 2017. For 2018, the means were 14.2 ± 2.92 with black light and 3.4 ± 0.84 without light. The data for the mean number of captured midges over the entire 3 year period for each species is found on table 2.1. There was a significant difference in the mean specimens captured for only 2 species, *C. bigutattus* and *C. stellifer,* for traps with light versus no light.

From the CDC traps with light a total of 1757 specimens from these 5 species in 286 pools were tested for BTV and EHDV by RT-qPCR; there were 11 BTV and 16 EHDV positive pools for an overall minimum infection rate (MIR) from all 5 species combined of 6.2 for BTV and 9.1 for EHDV. For the traps without light, there were 10 BTV positive pools and 2 EHDV positive pools out of 199 representing 709 specimens. The MIR of all 5 species for traps without light for BTV was 14.1 and for EHDV it was

2.8. The individual MIR's for each species for BTV and EHDV for traps with and without black light are provided in Table 2.3.

- Rotator Trap Study

A total of 701 specimens from 9 species of *Culicoides* were captured in the rotator trap: *C. arboricola, C. biguttatus, C. crepuscularis, C. debilipalpis, C. haematopotus, C. stellifer, C. variipennis, C. neopulicaris,* and *C. venustus*. Over 95% of the captured specimens were either *C. debilipalpis* or *C. stellifer*. The number of specimens collected at each of the time periods for each of the 5 species found to be PCR+ for this study are provided in Table 2.4; the number of collected specimens from the other 4 species that were PCR negative (*C. arboricola, C. biguttatus, C. variipennis, C. neopulicaris)* accounted for less than 3% of specimens captured in the rotator trap.

There was a significant difference (p=0.015) between the total specimens captured between 0600-0800h and 1800-2000h, 0600-0800h and 2000-2200h, and 0600-0800h and 2200-2400h. Sunrise was on average 0619h and sunset at 2005h and the average min and max temperatures in °C for each time period was 28-32, 26-28, 25-26, 24-25, 24-23, 23-23, 23-28. Over 40% of total specimens were captured from 0600-0800 with the average of 18.8 specimens compared to an average of 2.8 specimens collected in the 1800-2000 interval (Table 2.4). The time period when the most species (5 of 9) were collected was after sunset from 2000-2200h. There were 4 species (*C. arboricola, C. biguttatus, C. crepuscularis,* and *C. venustus*) that were caught from 0000h-6000h.

- Trap Comparison Study

There was no significant difference (p=0.122) in the overall mean number of specimens of *Culicoides* midges captured among all trap types (Table 2.5). Although the baffle trap

Table 2.1. The mean number of specimens captured from 11 species of *Culicoides* present in 2 different trap types baited with dry ice from 2016-2018 at the Bob R. Jones Idlewild Research Station near Clinton, La (DF=239, F=6.12, p=0.014, n=120).

	Mean # Specimens Captured/Trap-Night ± Standard Error										
CDC Trap	*C. arb**	*C. big***	*C. crep*	*C. vill*	*C. deb*	*C. ven*	*C.var*	*C. stel***	*C. neop*	*C. haem*	*C. fur*
black light	0.38 ± 0.14	1.12 ± 0.31 [A]	0.50 ± 0.25	0.07 ± 0.028	2.15 ± 0.54	2.70 ± 1.59	0.54 ± 0.15	9.06 ± 2.68 [A]	0.28 ± 0.10	0.23 ± 0.08	0.05 ± 0.08
no light	0.20 ± 0.076	0.27 ± 0.099 [B]	0.13 ± 0.05	0.05 ± 0.023	3.68 ± 1.36	0.11 ± 0.05	0.28 ± 0.08	1.90 ± 0.77 [B]	0.14 ± 0.05	0.12 ± 0.05	0.00 ± 0.00

**C. arb = C. arboricola, C. big=C. biguttatus, C. crep=C. crepuscularis, C.vill=C. villosopennis, C.deb=C. debilipalpis, C. ven=C. venustus, C. var=C. variipennis, C.stel=C. stellifer, C. neop=C. neopulicaris, C. haem=C. haematopotus, C. fur=C. furens*

**After testing by one –way ANOVA and Tukey's separation of means, values across columns followed by a different letter were significantly different ($P < 0.05$).

Table 2.2. Total number of specimens, BTV and EHDV positive pools using RT-qPCR, and total number of pools tested from 5 out of 11 species of *Culicoides* collected yearly from 2016-2018 using CDC traps with or without black light from June-November at the Bob R. Jones Idlewild Research Station near Clinton, La.

			EHDV positive pools		**BTV positive pools**		**Total pools tested**	
Year	Species	Total # specimens	black light	no light	black light	no light	black light	no light
2016	*C. crepuscularis*	31	0	0	0	0	2	1
	C debilipalpis	332	0	0	2	1	33	21
	C. stellifer	679	1	0	0	0	16	10
	C. haematopotus	19	0	0	1	0	34	21
	C. venustus	148	2	0	0	0	25	17
2017	*C. crepuscularis*	28	1	1	0	0	9	7
	C debilipalpis	230	1	0	0	2	21	18
	C. stellifer	334	3	1	2	2	10	6
	C. haematopotus	8	2	0	0	0	23	15
	C. venustus	100	5	0	2	1	17	12
2018	*C. crepuscularis*	16	0	0	1	0	7	2
	C debilipalpis	137	0	0	1	1	36	24
	C. stellifer	300	0	0	1	1	7	7
	C. haematopotus	15	0	0	1	1	28	22
	C. venustus	89	1	0	0	1	16	13
Total		2466	16	2	11	10	286	199

Table 2.3. Total number of specimens, BTV and EHDV positive pools, and MIR for 5 species of *Culicoides* captured in CDC traps with and without black light in June-November from 2016-2018 at the Bob R. Jones Idlewild Research Station near Clinton, La.

	Total Specimens Tested		# Positive Pools (MIR)			
Species	Black Light	No Light	EHDV Black light	EHDV No Light	BTV Black light	BTV No Light
C. crepuscularis	60	15	1(3.2)	1 (4.7)	1 (3.2)	0 (0.0)
C debilipalpis	258	441	1(3.2)	0 (0.0)	3 (9.5)	4 (18.7)
C. stellifer	1087	226	4 (12.7)	1 (4.7)	3 (9.5)	3 (14.0)
C. haematopotus	28	14	2 (6.3)	0 (0.0)	2 (6.3)	1 (4.7)
C. venustus	324	13	8 (25.4)	0 (0.0)	2 (6.3)	2 (9.4)

caught on average more than 3 times as many specimens of *Culicoides* than any other traps, there was a large variance and therefore there was no statistically significant difference in the mean number of specimens captured vs other traps. Of the 6 species captured, 3 species (*C. venustus, C. variipennis,* and *C. neopulicaris*) accounted for less than 2% and were not included in the analysis. For the other 3 species there were no significant differences in the mean number of midges captured in each trap type (Table 2.5).

There was a difference in the number of *Culicoides* species captured using the animal baited traps (drop trap, baffle trap, and direct animal aspirations) when compared to the CDC traps with or without light (Table 2.5). The CDC trap with light captured specimens from 7 species of *Culicoides*: *C. arboricola, C. debilipalpis, C. neopulicaris, C. pusillus, C. stellifer, C. variipennis* and *C. venustus*, and this was the only trap type that captured *C. arboricola* (only 1 specimen). During this trap comparison, the CDC trap without light captured specimens from 4 species: *C. debilipalpis, C. stellifer, C. variipennis,* and *C. pusillus,* but in the light vs no light study both *C. arboricola* and *C. venustus* but no *C. neopulicaris* were captured using this technique. The baffle trap method captured specimens from 6 species: *C. debilipalpis, C. neopulicaris, C. pusillus, C. stellifer, C. variipennis,* and *C. venustus.* The drop trap method caught specimens from 5 species: *C. debilipalpis, C. neopulicaris, C. pusillus, C. stellifer,* and *C. venustus*. Direct aspirations from the stanchioned calf yielded specimens from *C. debilipalpis, C. pusillus,* and *C. stellifer*. For each trap type, the majority of specimens were from *C. debilipalpis* ranging from 62% in the drop trap up to 94% in the direct aspiration. Specimens from *C. debilipalpis* accounted for over 85% of the total number of specimens captured from all traps. The next most abundant species in the study was *C. pusillus*

Table 2.4. The overall mean and total number of 3 species of *Culicoides* specimens captured per 2 hour time interval using a rotator trap over a 3 year period at the Bob R. Jones Idlewild Research Station near Clinton, La (DF=104, F= 2.77, p=0.015, n=15).

		Total Number of Specimens Captured				
Time Interval	Overall Mean ± SE *	*C. debilipalpis*	*C. crepuscularis*	*C. haematopotus*	*C. stellifer*	*C. venustus*
1800-2000h	2.80 ± 0.77^{A}	32	0	1	9	0
2000-2200h	3.53 ± 0.89^{A}	20	0	0	27	0
2200-2400h	4.53 ± 2.21^{A}	11	0	0	12	0
0000-0200h	5.93 ± 2.31^{AB}	39	0	0	0	1
0200-0400h	5.06 ± 1.96^{AB}	18	3	0	0	0
0400-0600h	6.06 ± 2.59^{AB}	22	5	0	0	0
0600-0800h	18.8 ± 7.32^{B}	265	0	0	17	0

* SE=standard error; After testing by one-way single factor ANOVA and Tukey's separation of means, values across columns followed by the same letter were not significantly different (P > 0.05).

Table 2.5. The mean number of specimens of 3 species *Culicoides* captured using 5 different trap types in 2014 at the Bob R. Jones Idlewild Research Station near Clinton, La (DF=39, F=1.96, p =0.122, n=8).

			Mean Number of Specimens Captured ± SE*		
Trap Type	Overall Mean ± SE*	Time	*C. pusillus*	*C. debilipalpis*	*C. stellifer*
Baffle trap	61.5 ± 29.89	1930-2045h	2.13 ± 1.22	58.50 ± 29.34	0.38 ± 0.26
Drop trap	19.1 ± 5.83	1930-2045h	6.13 ± 3.88	11.88 ± 5.13	0.75 ± 0.62
CDC black light	12.0 ± 5.53	1930-0700h	0.25 ± 0.25	9.63 ± 4.97	1.63 ± 0.98
CDC no light	16.5 ± 9.66	1930-0700h	3.38 ± 3.23	11.25 ± 6.24	1.88 ± 1.20
Direct aspiration	15.6 ± 5.32	1930-2015h	0.13 ± 0.13	12.63 ± 5.37	1.00 ± 0.42

*After testing by one-way single factor ANOVA there was no significant differences in the overall or species means (P > 0.05).

(8.8%) followed by *C. stellifer* (4.5%). The other 4 species (*C. arboricola, C. debilipalpis, C. neopulicaris, C. variipennis* and *C. venustus*) combined represented less than 2% of total specimens captured during the study. A small proportion of specimens of *C. stellifer* were captured in the 3 animal traps indicating this species was not highly attracted to the calf under the conditions of this study. In addition, abundance of *C.pusillus* was not well represented by black light or baffle traps (Figure 2.1).

2.4 Discussion

Over a 3-year trapping period, the CDC trap with black light and dry ice caught significantly (p=0.014) more *Culicoides* midges than the CDC trap with dry ice and no light. This is consistent with previous studies which have shown that by adding black light rather than incandescent to an insect trap, the total number of midges captured can increase by more than 10-fold (Rowley and Jorgensen 1967, Wieser-Schimpf et al. 1990). For this study, the total number of midges captured in the CDC traps with black light was over 2.5 times as many as CDC traps without light over 3 years. However, the number of specimens captured varied from year to year, which is typical for multiyear studies on members of the genus *Culicoides* (Sanders et al. 2019). Over the 3 year trapping period, the range for the yearly mean number of specimens captured in traps without light was 3.4-9.2 and for traps with black light 14.2-15.2. Therefore, when conducting trapping studies involving *Culicoides* midges and transmission it is advantageous to have multiple year field data due to yearly changes in weather or other variables.

For CDC traps with and without black light there were differences in the species captured; specimens of *C. furens, C. neopulicaris*, and *C. villosopennis* were only captured in CDC traps with black light. This is important information for conducting surveillance in an area of unknown species. Previous studies have utilized traps without

light to determine species of *Culicoides* present in areas experiencing outbreaks of bluetongue or epizootic hemorrhagic disease (Mands et al. 2004, Fassotte et al. 2008). We showed that traps without black light captured 27.3% of all specimens of *Culicoides* collected for both trap types combined as well as a lower diversity of species.

There was a general trend of more specimens of *Culicoides* to be captured in the rotator trap through time and as the temperature decreased (Table 2.4). Specimens of all species were not collected at all time periods, but more extensive studies would be required to show the nocturnal activity of all of the species. More specimens of both *C. debilipalpis* and *C. stellifer* were collected at sunrise from 0600-0800h than in the evening from 1800-2000h under the conditions of the study, and these 2 species are probable vectors of BTV and EHDV in Louisiana and other areas. The total number of specimens captured from 0600-0800h was more than 9 times for *C. debilipalpis* and 2 times more for *C. stellifer* than the number captured from 1800-2000h. These findings could have different applications. If animal trap studies aimed at finding species or specimens in different physiological status, such as nulliparous or gravid, that are not represented in light traps, then trials should be conducted at times when target species were most active. Therefore, conducting timed collections over the entire activity period of *Culicoides* spp. could be useful in timing animal trap studies. Furthermore, these data are relevant for deer farmers who conduct vector control in an attempt to decrease the chances of orbivirus transmission in their deer herds. The white-tailed deer farming industry in U.S. is growing and was estimated at 3 billion U.S. dollars in 2007 (Anderson 2007). Controlling adult midges that transmit BTV/EHDV by spraying insecticides at white-tailed deer farms is a common practice in the U.S. The data from this study show that the optimal time to spray in order to maximize control for specimens of *C.*

debilipalpis is at sunrise during the extremely hot summer days in Louisiana when the temperatures are cooler in the morning. Fisher et al. (2015) showed that spraying insecticides at lower temperatures in the morning is most effective, especially for reaching low flying insects because the thermals in high heat areas prevent ultra-low volume (ULV) insecticide sprays from reaching the ground.

There was no significant difference in the mean number of *Culicoides* specimens among all five trap types which suggests that light trap catches are a good representative of the abundance of *Culicoides* midges at the trapping location for the current study. More importantly, we did not capture any *Culicoides* species in the animal baited traps that we did not catch in the light traps. Our results differ from those Viennet et al. (2011) who showed that 2 species of *Culicoides* (*C. subfasciipennis and C. picturatus*) were captured in animal baited traps that were not captured in light traps in western France although they also captured 6 species of *Culicoides* in the light traps that were not captured in the animal trap.

Several studies have emphasized the importance of utilizing different trap types in areas of orbivirus transmission especially when conducting vector surveillance because of known differences in species diversity captured using different trap techniques (Mayo et al. 2012, McDermott et al. 2015). Carpenter et al. (2008) captured specimens of *C. chiopterus* in animal baited traps but not in light traps and stressed the importance of comparing alternative/additional methods of surveillance in different locations. Under the conditions of this study we showed that the animal baited trap methods (drop trap, baffle trap, or direct aspirations from calves) did not capture any different species of *Culicoides* than the light traps indicating that long term trapping studies for abundance of difference species utilizing CDC light traps are valid at the study site and likely other

similar habitats. One explanation for the difference in our results compared to previously mentioned studies is that the studies were conducted in different locations with different species of *Culicoides*, and different types of animal production systems. The ecology and host-seeking behavior of different *Culicoides* species likely varies greatly among different habitats.

For each animal baited trap there was no significant difference in the number of specimens captured per time period, but there were differences in the number of species captured. There are biases to consider when using live animal hosts to capture insects which include changes in insect behavior in response to a restrained animal, the design and presentation of the trap and trapping protocol, or influence of human collectors and their associated cues (Mullens and Gerry 1998). There are distinct differences in the factors that were involved in each of the animal baited traps we utilized. The direct aspirations from the calf included a human collector, which may attract or repel certain midges depending on the species. For example, specimens of *C. impunctatus* were shown to be attracted to certain human odors but repelled by others after performing choice tests in the lab (Logan et al. 2009). For the drop trap, there were no humans near the trap for a period of time when midges were allowed to fly in, land, and feed openly on the calf with no barriers. However, the efficacy of drop traps is limited by the feeding time of different species. One study compared feeding times of *C. variipennis* on ponies versus the ingestion of *O. cervicalis* microfilarie and reported some specimens of *C. variipennis* took over 18 minutes to take a blood meal (Higgins 1988). Therefore, frequent collections would be required to accurately portray the diversity of species feeding on the animal. The baffle trap has several barriers or obstructions for insect entry into the trap; examples of these challenges are the height of the baffles and lack of a

visual host. Once specimens are in the baffle trap, the need to address feeding time of different species is negated. However, certain midge species are exophillic meaning they are reluctant to enter any enclosure or cage to attack their host (Blanton and Wirth 1979). All of the above mentioned differences in the animal traps could affect the diversity in *Culicoides* species captured by the different methods. The results from the trap type study showed that *C. debilipalpis*, which is the probable primary vector of BTV in Louisiana, was captured using all 5 trap types (Figure 2.1). The baffle trap and the CDC trap with light caught all 6 species and the CDC trap without light only caught 3 species. Therefore, the baffle trap and CDC with light have value in estimating species diversity under the conditions of the study.

For the comparison of light versus no light on the capture of BTV PCR positive specimens, there was a higher MIR overall for the combined 5 species in the number of BTV infected pools in CDC traps without black light baited with dry ice versus CDC traps baited with dry ice and black light. McDermott et al. (2015) showed that no BTV infected *C. sonorensis* midge pools came from UV only light traps early and late in the transmission season and concluded that infected midges may be repelled by light because large amounts of BTV were found in the eyes. However, their study only focused on specimens of *C. sonorensis*, which were not present at the sites for the current study or many other locations without intensive animal agriculture. Similar to their results, we found the minimum infection rates for BTV were higher in CDC traps with CO_2 with no light for all 5 species combined, but our results indicate there are differences in BTV MIR at the species level. For BTV, 2 species (*C. crepuscularis* and *C. haematopotus*) had lower infection rates in the traps without light, which is in disagreement with the

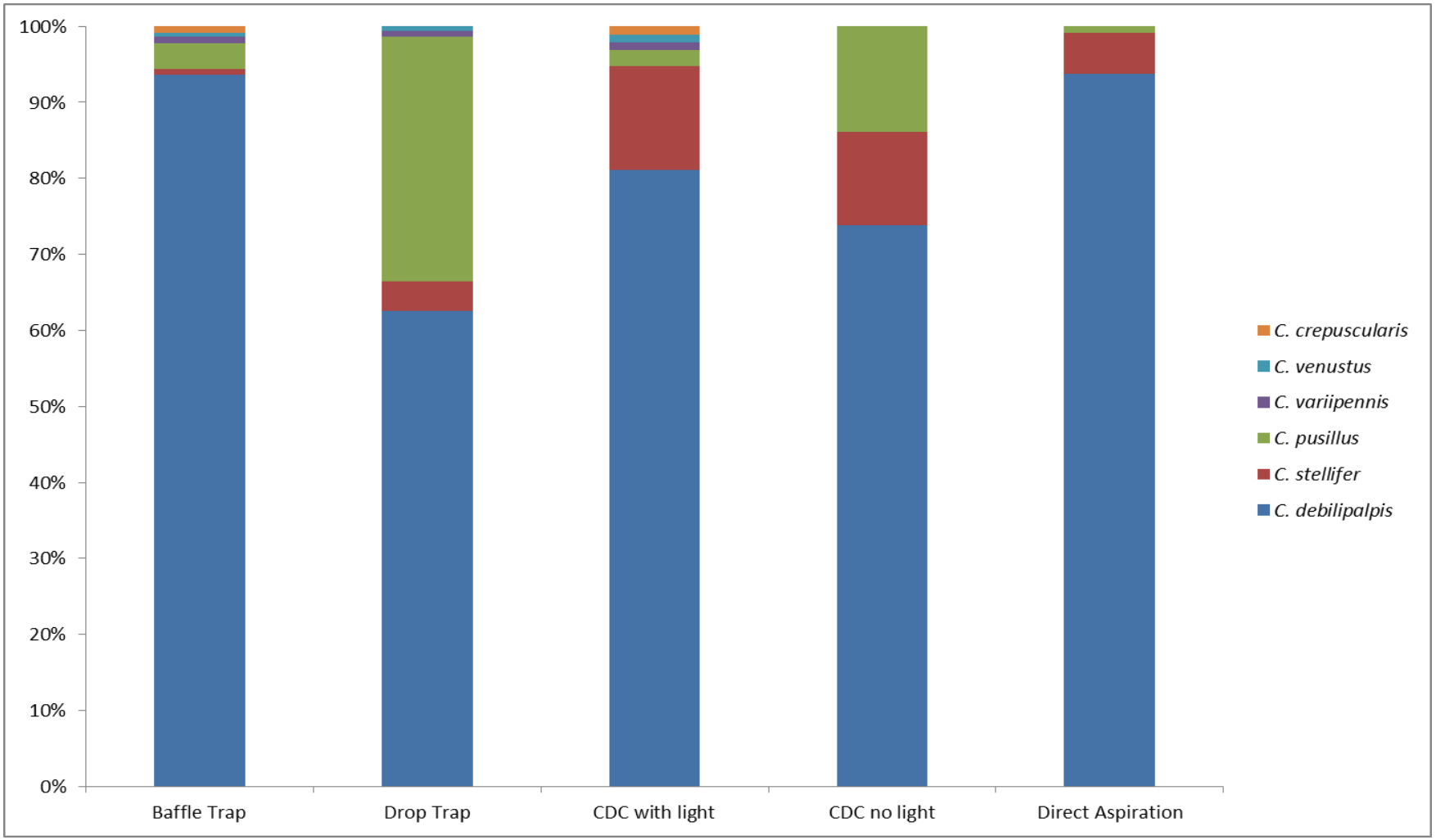

Figure 2.1. Species percent composition of *Culicoides* midges captured in 5 different trap types (baffle trap, drop trap, CDC trap with black light, CDC trap without light, and direct aspirations from a calf) in 2014 at the Bob R. Jones Idlewild Research Station near Clinton, La.

previous study mentioned above. On the other hand, for EHDV the MIR was much higher for all species combined in the traps with black light versus the traps without black light. Only *C. haematoptus* had a higher EHDV infection rate for the traps without light (Table 4). The results showed 4 species (*C. crepuscularis, C. debilipalpis, C. stellifer,* , and *C. venustus*) had significantly higher EHDV MIR in the traps with black light indicating a difference in trap catches for EHDV infected midges as compared to BTV. For BTV or EHDV infected *Culicoides* midges, there may be differences at the species level on the effects of light repellency and futures studies are needed to clarify this phenomena. Given the differences in species diversity between traps with and without light and the potential differences in behavior of infected midges related to infection of the eyes, the use of with and without lights is warranted in studies aimed at vector incrimination for orbiviruses.

Knowledge of host-seeking behavior and feeding success for adult female specimens of different *Culicoides spp.* is important information to gain for vectors of BTV and EHDV for assessing transmission risk. Since there are over 4000 species of *Culicoides*, host seeking behavior and feeding success cannot be generalized among species or habitats. One study showed octenol + phenol and octanol + CO_2 increased collections by 100 times over CO_2 alone for *C. furens* (Kline et al. 1990*)*. Tanner and Turner (1974) found that, after testing different animal baited traps, height above ground level and size of the host animal was more important than the type of host. It is known that variables such as time of day, trap height, amount of CO_2, type of light, location of trap, proximity of animals can affect results on midge surveillance and our studies are consistent with this. Although many female *Culicoides* midges will likely feed on the nearest available host, each species may have a slightly different search pattern. Our

study and others continue to emphasize that there is no one size fits all approach to the study of epidemiology of orbiviruses.

Each of the 3 studies in this paper add value to selecting trapping techniques to detect presence of *Culicoides* species in given areas, utilization of trap types for orbivirus detection in midges, and activity periods for species present. Our data support other studies that indicated that when searching for *Culicoides* midges infected with orbiviruses it is advisable to use traps both with and without light. Once it is determined which species are captured with light traps, use of a timed collection, such as the rotator trap, can aid to determine the best times to use other trap types such as animal traps as well as identify optimal times for control efforts. The three different animal traps used in this study offer different value depending on the objectives of the study. Direct aspirations from animals offers the least value for vector abundance because of human interference but is valuable when attempting to determine biting rates for vector species. The baffle trap is highly useful when trying to collect blood fed midges if the species of interest will enter an enclosure through baffles to attack a host. The drop trap is the least biased animal trap because there is no human interaction and the insects are allowed to naturally feed on the animal. However, the window of time for midge feeding is short and the timing must be precise to capture the targeted species.

When conducting entomological surveillance in a new area with orbivirus transmission, our studies indicate that traps with and without light should first be utilized to capture the most species/specimens of BTV/EHDV infected midges. Subsequently, activity periods of the species of interest could be determined using timed trapping techniques which would identify the activity periods of species interest to conduct more intensive studies using techniques of value to the objectives of the study.

CHAPTER 3. PROSPECTIVE STUDY OF EPIZOOTIC HEMORRHAGIC DISEASE VIRUS AND BLUETONGUE VIRUS TRANSMISSION IN CAPTIVE RUMINANTS

3.1 Introduction

Epizootic hemorrhagic disease virus (EHDV) and bluetongue virus (BTV) are members of the genus *Orbivirus* and cause hemorrhagic disease (HD) in white-tailed deer (*Odocoileus virginianus*). Clinical signs and symptoms of infection include cyanosis, internal hemorrhaging, lameness, high fever, and dehydration. In livestock, such as cattle, EHDV causes epizootic hemorrhagic disease (EHD) and BTV causes bluetongue (BT) disease. Cattle infected with BTV normally do not develop clinical disease, but in some cases symptoms such as heavy salivation, weight loss, coronitis, and stillbirth or abortion can occur (Elbers et al. 2008). Cattle are reservoir hosts for BTV due to the subclinical disease and prolonged viremia of up to 140 days post-infection (MacLachlan et al. 1994); however, other research concluded that infectious BTV persists for less than 60 days in cattle (Singer et al. 2001). The duration of EHDV viremia in cattle has not been well studied; but in one study Gibbs and Lawman (1977) inoculated 6 calves with EHDV-2 and isolated the virus from 4 of the calves for up to 28 days post infection and from 1 calf for 50 days post infection. Outbreaks of EHD or BT occur when susceptible animals are introduced into regions where these viruses are endemic or when the viruses spread into immunologically naïve ruminant populations (Maclachlan et al. 2009). For example, there was an outbreak of BT disease caused by BTV in Europe in 2006-2007 which resulted in the death of tens of thousands of sheep and affected over 30,000 farms. The outbreak was caused by BTV-8, a serotype that had been previously unknown in the Europe (Elbers et al. 2008). Similarly, there was a large outbreak of HD among white-

tailed deer, caused by EHDV-2, in the U.S. in 2007 in northern states where infections in deer had rarely been observed previously (Ruder et al. 2015).

Currently there are 26 recognized serotypes of BTV and 7 serotypes of EHDV worldwide (Campbell and George 1986, Anthony et al. 2009, Maan et al. 2012). In the U. S., five bluetongue virus serotypes (2, 10, 11, 13, and 17) were present prior to 1998 with an additional 10 serotypes (1, 3, 5, 6, 9, 12, 14, 19, 22 and 24) more recently reported in the southeastern United States from both cattle and deer (Maclachlan et al. 2013). EHDV serotypes 1 and 2 are historically endemic in the U.S and EHDV-6 was first reported in 2006 (Ruder et al. 2017).

When present, the primary vector for BTV and EHDV in the U.S. is considered to be *Culicoides sonorensis* Wirth and Jones. This species is a member of the *C. variipennis* complex, which was shown by Holbrook et al. (2000) to be three separate species: *C. variipennis* Coquillett, *C. occidentalis* Wirth and Jones, and *C. sonorensis*. Specimens of *C. variipennis* and *C. occidentalis* are considered to be refractory to BTV and not considered competent vectors of orbiviruses. Several studies have shown female *C. sonorensis* to be competent vectors of different serotypes of BTV and EHDV (Foster et al. 1977, Mecham et al. 1990, Wittmann et al. 2002). Since specimens of *C. sonorensis* are competent vectors of multiple serotypes of different orbiviruses, the presence/absence of this species is important when conducting insect surveillance during an outbreak of BT or EHD.

The larval habitats of *C. sonorensis* have been described as mucky substrates usually consisting of highly organic runoff; these habitats are primarily associated with intensive agriculture practices. For example, several studies have reported high numbers of *C. sonorensis* larvae collected on the edge of wastewater dairy lagoons (Mullens and

Rodriguez 1988, Schmidtmann et al. 2000). On beef cattle farms with pastured animals, optimal larval habitats for *C. sonorensis*, such as the edge of a wastewater lagoon, often are not present. Therefore, it is important to determine the species in local populations of *Culicoides* which are potential vectors of BTV and EHDV in areas experiencing transmission of these viruses in the absence of *C. sonorensis.* In Florida, *C. insignis* Lutz is the considered vector of BTV-2 as the virus was isolated from field caught specimens in areas where *C. sonorensis* was absent. In addition, specimens of *C. insignis* were shown to transmit BTV-2 to sheep after intrathoracic inoculation (Greiner et al. 1985, Tanya et al. 1992) . Similarly, Becker et al. (2010) found specimens from four different species of *Culicoides* (*C. haematopotus* Malloch, *C. crepuscularis* Malloch, *C. debilipalpis* Lutz, and *C. furens* Poey) to be RT-PCR positive for BTV in Louisiana in an area of active BTV transmission where *C. sonorensis* was absent. In addition, McGregor et al. (2018) trapped specimens from 11 species of *Culicoides*, but no *C. sonorensis*, at a big game preserve in Florida where BTV and EHDV are transmitted.

One of the largest epizootics of HD recorded in U.S. history occurred in 2012. South Dakota Game, Fish, and Parks gave refunds for hunting licenses due to the high level of deer mortality. The state of Michigan recorded 15,000 dead white-tailed deer attributed to HD, and in Nebraska a 30% decline in white-tailed deer harvest was reported (Schrauben 2013). Furthermore, wildlife experts reported 2012 to be one of the worst HD outbreaks in Kansas, and in Missouri, over 10,000 deer deaths were reported (Flinn and Sumners 2013). In spite of the impact of HD on the reported reduction of wild deer populations, little information is available on the vectors associated with this epizootic.

In 2012, Louisiana deer farmers reported more deaths attributed to HD than had been observed in previous years (personal communication, James LaCour). In this paper, we report the results of a prospective study conducted in 2012 at a location with captive white-tailed deer and cattle. We surveyed ceratopogonid populations, tested cattle and deer for development of a detectable antibody response to BTV and EHDV, and used reverse transcriptase quantitative Polymerase Chain Reaction (RT-qPCR) assays to detect the presence of BTV and EHDV nucleic acid in insects as well as in tissues and whole blood collected from deer and cattle.

3.2 Material and Methods

The study was conducted at the Louisiana State University Agricultural Center Bob R. Jones Idlewild Research Station (BJIRS) near Clinton, Louisiana (30.817954N, -90.97324W). Yearly, a reproductive herd of approximately 80 captive white-tailed deer is maintained among 11 fenced enclosures, ranging from approximately 0.1 to 1.8 hectares, and a reproductive herd of approximately 50 adult crossbred beef cattle is maintained among six pastures ranging from 18 to 30 hectares. There was no attempt to provide a buffer zone between the cattle and deer with one of the cattle pastures being within 100 meters of the deer enclosures. The penned white-tailed deer is a closed herd, although the 830 hectare facility is home to wild white-tailed deer; however the cattle herd is not closed. In January 2012, 21 crossbred cows were purchased and transferred from a livestock facility in Baton Rouge, La. The origin of the majority of the individual cows before they were consolidated at the facility in Baton Rouge was not available. However, by tracing the USDA tag numbers through the Texas Department of Agriculture, some of the cattle were known to have originated from five different counties in Texas including

Medina, Burleson, Limestone, Wood and Red River where BTV-10, 11, 12, 13, 17 and EHDV-1, 2, and 6 have been reported (Stallknecht et al. 1996, Allison et al. 2012)

- Serology

In September 2011 (pre-season 2012), blood samples were obtained from all cattle (35 total) and white-tailed deer (64 total) at the BJIRS; serum was harvested and stored at -20°C. Blood samples were collected in April 2012 from the 21cattle that were transferred to the research station in January, and the serum was stored at -20°C. Serum samples were collected from the same cattle and white-tailed deer listed above that remained in November 2012 (post season). Also, serum samples were collected from 16 white-tailed deer that were hunter-killed on November 12, 2012 at Camp Avondale, a wildlife management area less than 8 km from the BJIRS. All serum samples were screened for antibody to EHDV and BTV, separately, using the agar gel immunodiffusion (AGID) test (Veterinary Diagnostic Technology, Inc., Wheat Ridge, CO) following the manufacturer's recommended procedures. If the serum sample tested contains antibodies to BTV/EHDV then they bind to the antigens used in the assay forming an interlaced antigen-antibody complex that precipitates in the agar and a clear line is visible (Afshar et al. 1989). The AGID test was performed on cattle and deer serum for both EHDV and BTV in attempt to capture all seroconversions because cross reactivity has been described (Stallknecht et al. 1991).

- Deer Death Records and PCR Diagnostics

The white-tailed deer herd was monitored daily by a deer manager and pen location, tag identification number, and death date were recorded for adult and fawn white-tailed deer from June through November 2012 at the Idlewild Research Station. Whole blood from live cattle and deer, and tissues such as spleen, tonsils, and mandibular

salivary glands were harvested from dead deer and submitted to the Texas A & M Veterinary Medical Diagnostic Laboratory, College Station, Texas. Homogenates were prepared by crushing 0.10-0.20g of tissue in a 2.0mL Eppendorf vial using a plastic pestle and a pestle motor (model #749540). Then, 900 µL of "BA-1 buffer" consisting of Hanks M-199 salts, 0.05 M Tris, pH 7.6, 1% bovine serum albumin, 0.35g/L sodium bicarbonate, 100 U/mL penicillin, 100ug/mL streptomycin, and 1µg/mL fungizone was added to each vial. Viral RNA was isolated from the blood and tissue samples (50 µl blood sample, or 50 µl tissue homogenate; 10% w/v in 1X phosphate buffered saline) using the KingFisher96 automated particle processor and the MagMAX™-96 Viral RNA Isolation Kit, as previously described (Schroeder et al. 2013). BTV and EHDV multiplex RT-qPCR utilized the PathID Multiplex OneStep kit from Applied Biosystems/Life Technologies. Total RNA was denatured by heating the extracted nucleic acid to 95°C for 3 minutes. Master mix preparation and cycling conditions were as previously described (Schroeder et al. 2013). Samples were considered EHDV positive when cycle quantification (Cq) values were less than 40 cycles and BTV positive when Cq values were 36 cycles or less (Schroeder et al. 2013). Samples that were EHDV positive were serotyped using a gel-based multiplex reverse transcriptase polymerase chain reaction, or RT-PCR assay, using specific EHDV primers (Table 3.1). Each RT-PCR reaction contained 10.2 µl RNase-free water, 5.0 µl 5X RT-PCR buffer (Qiagen OneStep RT-PCR buffer), 1.0 µl 25 mM MgCl2, 0.8 µl 10 mM dNTP mix, 1.0 µl RT-PCR enzyme, 0.5 µl RNase inhibitor (13 units/µl), and 0.25 µl of each forward (F1, F2, F6) and reverse (R1, R2, R6) serotyping primer (0.5 µg/µl working concentration of each primer). Denatured template (5 µl) was mixed with 20 µl of the RT-PCR mixture and amplified using a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA).

The RT-PCR cycling conditions were as follows: 50°C for 50 min, 95°C for 10 min, 45 cycles of amplification at 95°C for 20 sec, 60°C for 30 sec and 72°C for 45 sec, with a final 5 min extension at 72°C, followed by refrigeration. Amplified PCR products were visualized on a 1.5% agarose gel. Amplified DNA products (5 µl) were mixed with 2 µl DNA loading buffer containing GelRed DNA stain (Biotium Inc., Hayward, CA), loaded onto the gel and run at 70-90 volts for 45 min. Bands were visualized under UV light. Only those samples containing bands of the correct size (389 bp for EHDV-1, 246 bp for EHDV-2 and 500 bp for EHDV-6) were considered positive (Table 1). Samples (blood or tissue) that were RT-qPCR positive for BTV were submitted to the National Veterinary Services Laboratory (NVSL) in Ames, Iowa for BTV serotyping.

- Insect Trap Study and PCR

From January through December 2012, eight miniature Centers for Disease Control (CDC) black light traps (model 512; John W. Hock Co., Gainesville, FL) baited with 2 kg of dry ice in igloo containers were hung approximately 1.5 m above ground at sites greater than 50 m apart. Six-volt 20 amp hour rechargeable batteries were used to energize the traps. The trap sites were set up in a 1km radius encompassing different habitats occurring among the research station. Three of the sites were within 20m of captive white-tailed deer pens where deer were present year round. Two of the sites were within 10m of cattle pastures where cattle were present all year. Two sites were in forested areas among hardwood and pine trees, and one site was between the edge of a 20 hectare freshwater lake and the deer pens. The traps were deployed before dusk and collection containers were retrieved after sunrise. Initially, traps were deployed once or twice a month for general surveillance purposes; however, weekly trappings were

Table 3.1. Sequence of primers and probes used for the RT-qPCR multiplex assay and list of primers used for traditional gel-based PCR serotyping assay for EHDV and BTV.

Primers ID	Sequence (5′–3′) and Reporter Dye	Target Region	Amplicon Size
Bluetongue Virus			
BTV-Forward	TGGAYAAAGCGATGTCAAA		
BTV-Reverse	ACATCATCACGAAACGCTTC	NSP3	96bp
BTV-probe	FAM-ARGCTGCATTCGCATCGTACGC-BHQ1		
Epizootic Hemorrhagic Disease Virus			
EHD-Forward	ACWGGVATCATGTTTGAGCT		
EHD-Reverse	TTCATAACCGCACCTTCATC		
EHD-probe 1	VIC-TCATCACACATCGGC-MGB	NSP1	110bp
EHD-probe2	VIC-TCTCGGCATATGCGAGC-MGB		
Serotype Primers for EHDV and BTV			
EHDV1-Forward	AATAGGCGATGTTGATCGACATC		
EHDV1-Reverse	TTCGAAACCTCGCTTGCAT	VP2	389bp

(table cont'd)

Primers ID	Sequence (5′–3′) and Reporter Dye	Target Region	Amplicon Size
EHDV-2 Forward	TGGTGAAAATACGGTGGTATATAACC	VP2	246bp
EHDV2-Reverse	GTTCAAATTCATCTGGGCTCATACT		
EHDV6-Forward	ATAACGAACAGGGAGCCKTATAAAA	VP2	500bp
EHDV6-Reverse	CCAAACTTCTCAGTAGCATACAACAT		
BTV12-Forward	AAGTGGGATGCGATCATGG	VP2	717bp
BTV12-Reverse	CCTTCCGGGTAGCATATGTAG		

* FAM = 6-carboxyfluorescein; BHQ1 = black hole quencher 1; MGB = minor groove binder; NSP = non-structural protein; NA = not applicable.

initiated following confirmation of EHDV positive tissues from the first deer to have died within the surveillance area. There was a total of 212 trap-nights during the entire year of 2012. Nets containing captured insects were placed into a cooler containing 5-10 kg of dry ice and immediately transported back to the laboratory and stored at -80 º C. Subsequently, the insects were sorted using a dissecting microscope and a chill table (BioQuip®, Gardena, CA). Ceratopogonids were sorted into genus by examining the wing venation, number of antennal segments, and maxillary palps using the Manual of Neartic Diptera (1981) as a reference. Members of the genus Culicoides were sorted to species through examination of wing patterns using the keys of Blanton and Wirth (1979) as a reference; voucher specimens were confirmed by mounting, dissecting and clearing followed by examination of the spermathecae.

Field-collected midges that were non-blood fed from the genus *Culicoides* were pooled by species, site, and date and placed into Eppendorf 2.0mL Safe-Lock tubes (Cat. No 022363352). Pools of at least 5 midges up to a maximum of 50 midges were homogenized in 900 µL BA-1 buffer, described above, with 2 stainless steel bb's at 50 hz for 10 minutes using the Tissuelyser II by Qiagen; viral RNA was isolated from the midge pools and RT-qPCR was performed as described above.

3.3 Results

- Serology

Of the 21 cattle that were transferred in 2012, 13 were seropositive for BTV or EHDV upon arrival: 6 out of 8 seronegative animals seroconverted for BTV or EHDV during the 2012 HD transmission season (Table 3.2). Of the 35 resident cattle on the farm, 14 were seropositive for BTV or EHDV pre-season and 13 of 21 (or 62%) seroconverted for BTV or EHDV. In total, the seroprevalence of BTV or EHDV antibody in the resident cattle increased from 31.4% to

Table 3.2. Serology results from 2012 pre and post vector season for the combined BTV and EHDV agar gel immunodiffusion test of serum samples collected from cattle and penned white-tailed deer in 2012 at the Bob R. Jones Idlewild Research Station and wild deer from Sherburne Wildlife Management Area.

	Total Animals	% BTV/EHDV Seropositive		# Animals Seroconverted
		PRE	POST	
Imported Cows	21	61.9	90.5	6 of 8
Resident Cows	35	40.0	77.1	13 of 21
Captive White-tailed Deer	64	12.5	40.6	18 of 58
Wild White-tailed Deer	16	N/A	87.5	N/A

over 75% post HD transmission season. Of the 64 white-tailed deer from BJIRS that were tested both pre and post season, 18 of 58 seroconverted for EHDV or BTV. Overall, the seroprevalence for EHDV or BTV in white-tailed deer increased from 12.5% pre-season to over 40% post season. Of the 16 hunter-killed deer from Camp Avondale, 14 were seropositive for BTV or EHDV (Table 3.2).

- Deer Death Records and PCR Diagnostics

A total of 25 adult and 54 fawn white-tailed deer died from June through November 2012. Samples of spleen, parotid salivary gland, tonsil, retropharyngeal lymph node, or whole blood were obtained from 19 adults and 4 fawns and placed into 50mL Eppendorf vials containing 25mL of BA-1 buffer, described above. Necropsies performed on most of these deer revealed subcutaneous petechial hemorrhage with dependent xanthochromic edema, mild to severe oral ulceration (lingual and mucosal) with or without secondary infection, and ruminal ulcerations. Of these, 18 adults and 2 fawns were RT-qPCR positive for BTV or EHDV (Table 3.3). Of the 18 adults, 7 were positive for EHDV-6, 5 were positive for BTV-12, one was positive for EHDV-1, and one was positive for both EHDV-6 and BTV-12 (Table 3.4). The RT-qPCR positive samples from the 2 fawns were not serotyped. There was one adult deer that survived an EHDV-6 infection which was confirmed from a blood sample.

In September 2012, an Angus bull exhibited clinical signs of BT or EHD including excessive salivation, weight loss, and lameness; the animal was positive for both EHDV-6 and BTV-12 (Table 3.5). A blood sample was collected on November 2, 2012 from one of the cows that was transferred to the farm in April 2012 and found to be positive for BTV-12. On November 16, 2012, blood was collected from 10 of the additional transferred cattle as well as from the previously positive bull;

Table 3.3. Individual white-tailed deer deaths at the Bob Jones Idlewild Research Station confirmed by RT-qPCR for EHDV or BTV in 2012 relative to date, virus, serotype, location, and age of deer, and Cq value from spleen, blood, or tonsil.

Deer #	Death Date	Virus	Serotype	Location	Age in years	Cq Value
Pink 152	8/8/2012	EHDV	6	Pen 4	1	22.8
Pink- 144	8/15/2012	EHDV	1	Pen 4	1	19.4
White-34	8/25/2012	EHDV	6	Pen 1	8.5	27.87
Brown-37	8/25/2012	EHDV	6	Pen 2	4.5	22.53
White-55	8/25/2012	EHDV	N/A	Pen 3	1.5	30.30
Pink-151	8/30/2012	EHDV	6	Pen 4	1.5	27.5
Brown-71	9/6/2012	BTV	N/A	Pen 1	0.08	35.2
Red-50	9/6/2012	BTV	N/A	Pen 2	2.5	25.2
Pink-101	9/6/2012	BTV/EHDV	12/6	Pen 4	1.5	38.2 , 24.5
Red 55	9/18/2012	BTV	12	Pen 1	2	27.1
Brown-43	9/19/2012	EHDV	N/A	Pen 1	0.17	39.5
Pink-113	9/22/2012	BTV	12	Pen 4	1.5	20.1
White 50	9/22/2012	EHDV	6	Pen 1	8.5	24.0
Pink 130	9/27/2012	BTV	12	Pen 4	1.5	21.1
Pink 121	10/2/2012	EHDV	6	Pen 4	1.5	23.8
Brown 4	10/26/2012	BTV	12	Pen 1	4.5	18.6
Pink-117	10/29/2012	BTV	N/A	Pen 4	1.5	15.9
Pink 135	11/1/2012	BTV	12	Pen 4	1.5	20.5
White 85	11/26/2012	BTV	N/A	Pen 3	1.5	23.5

Table 3.4. Confirmed hemorrhagic disease in white-tailed deer at the Bob R. Jones Idlewild Research Station from 2012 by virus type, time period, RT-qPCR Cq value range and tissue type. Cq values of 36 or less is positive for BTV and Cq values of 40 or less is positive for EHDV.

IDLEWILD WHITE-TAILED DEER HERD			RT-qPCR Cq VALUE			
VIRUS TYPE	**n**	**DATE DIAGNOSED**	**SPLEEN**	**BLOOD**	**TONSIL**	**SALIVARY GLAND**
EHDV – 1	1	08/15/2012	22.8	NA	25.3	NA
EHDV – 6	8	08/08/2012 – 10/02/2012	22.5– 27.9	35.3	30.4 – 32.7	29.2 – 31.9
EHDV NO TYPE	2	08/25/2012 – 09/19/2012	30.3	NA	35.0 – 35.6	NA
BTV – 12	6	09/18/2012 – 11/01/2012	16.0 – 27.1	NA	21.8 – 30.6	22.3 – 28.1
BTV NO TYPE	3	09/06/2102 – 11/07/2012	25.2 – 38.2	23.5	27.2– 35.0	27.4 – 32.6

Table 3.5. Confirmed bluetongue and epizootic hemorrhagic disease in cattle at the Bob R. Jones Idlewild Research Station from 2012 by virus type, time period and relative RT-qPCR cycle quantification (Cq) value range from whole blood. Samples with Cq values equal to or less than 36 are positive for BTV and Cq values less than 40 are positive for EHDV.

VIRUS TYPE	n	DATE DIAGNOSED	Cq value
EHDV – 2	1	11/16/2012	33.5
EHDV – 6	4	09/26/12 – 11/16/2012	35.5 - 35.9
BTV – 12	11	09/26/12 – 11/16/2012	24.5 – 33.8

samples were tested for EHDV and BTV via RT-qPCR and serotyped using traditional gel-based RT-PCR. Eleven of twelve animals (the bull and 10 of 11 transferred cows) were positive for BTV-12; additionally, four of the twelve tested were also positive for EHDV-6 (Table 3.5). One animal was positive for BTV-12, EHDV-6 and EHDV-2. On April 11, 2013 (147 days from the original RT-qPCR test) five animals were still positive for BTV-12 (Cq<36). One animal remained BTV-12 positive (Cq<36) for 175 days after the first RT-qPCR BTV positive result.

- Insect Trap Study and PCR

A total of 1,329 specimens representing 14 species of Culicoides were captured from January to December 2012: *C. arboricola* Root and Hoffman, *C. biguttatus* Coquillet, *C. crepuscularis* Malloch, *C. paraensis* Goeldi, *C. hinmani* Khalaf, *C. variipennis* Coquillett, *C. villosipennis* Root and Hoffman, *C. venustus* Hoffman, C. *nanus* Root and Hoffman , *C. furens* Poey , *C. neopulicaris* Wirth, *C. debilipalpis* Lutz, *C. stellifer* Coquillet, and *C. haematopotus* Malloch. From August until November, three species (*C. debilipalpis*, *C. stellifer*, and *C. crepuscularis*) accounted for over 86% of *Culicoides* specimens captured. In September alone, when 50% of confirmed BTV or EHDV-associated mortalities in deer occurred, over 90% of the specimens captured were

one of these three species (Figure 3.1). There was a large peak of *C. biguttatus* collected in the beginning of the year (March- May), but no more specimens were captured throughout the remainder of the year (Figure 3.2). There was a peak of *C. stellifer* in May and in October indicating that this species is a multi-voltine species in Louisiana. In 2012, 109 pools of 418 midges representing 10 species of Culicoides captured from August 10- December 19 were screened for BTV and EHDV. A total of three pools tested positive for BTV using RT-qPCR; none of the pools were positive for EHDV. The BTV positive pools came from three different species: *C. crepuscularis*, *C. debilipalpis*, and *C. stellifer*. Two of the BTV positive pools (*C. crepuscularis* and *C. stellifer*) were collected during the August 22 collection and the other pool (*C. debilipalpis*) from the September 18 collection. The Cq value for the *C. crepuscularis* (1 specimen) positive pool was 33.2 and for both the *C. debilipalpis* (3 specimens) and *C. stellifer* (4 specimens) positive pools, the Cq = 35.0.

3.4 Discussion

The RT-PCR results from tissues or blood collected from white-tailed deer and cattle in 2012 confirmed transmission of EHDV-1, EHDV-2, EHDV-6, and BTV-12 at the BJIRS. These are believed to be the first confirmed cases of BTV-12 and EHDV-6 from Louisiana (Ruder et al. 2017). The first report of BTV-12 in the U.S. was in 2008 from cervids in Texas; however, there have been no reports linking this serotype to an outbreak of HD. The first isolation of EHDV-6 in the U.S. was in 2006 (Allison et al. 2010). Stallknecht and Phillips (2012) reported that 2012 was the first year that EHDV-6

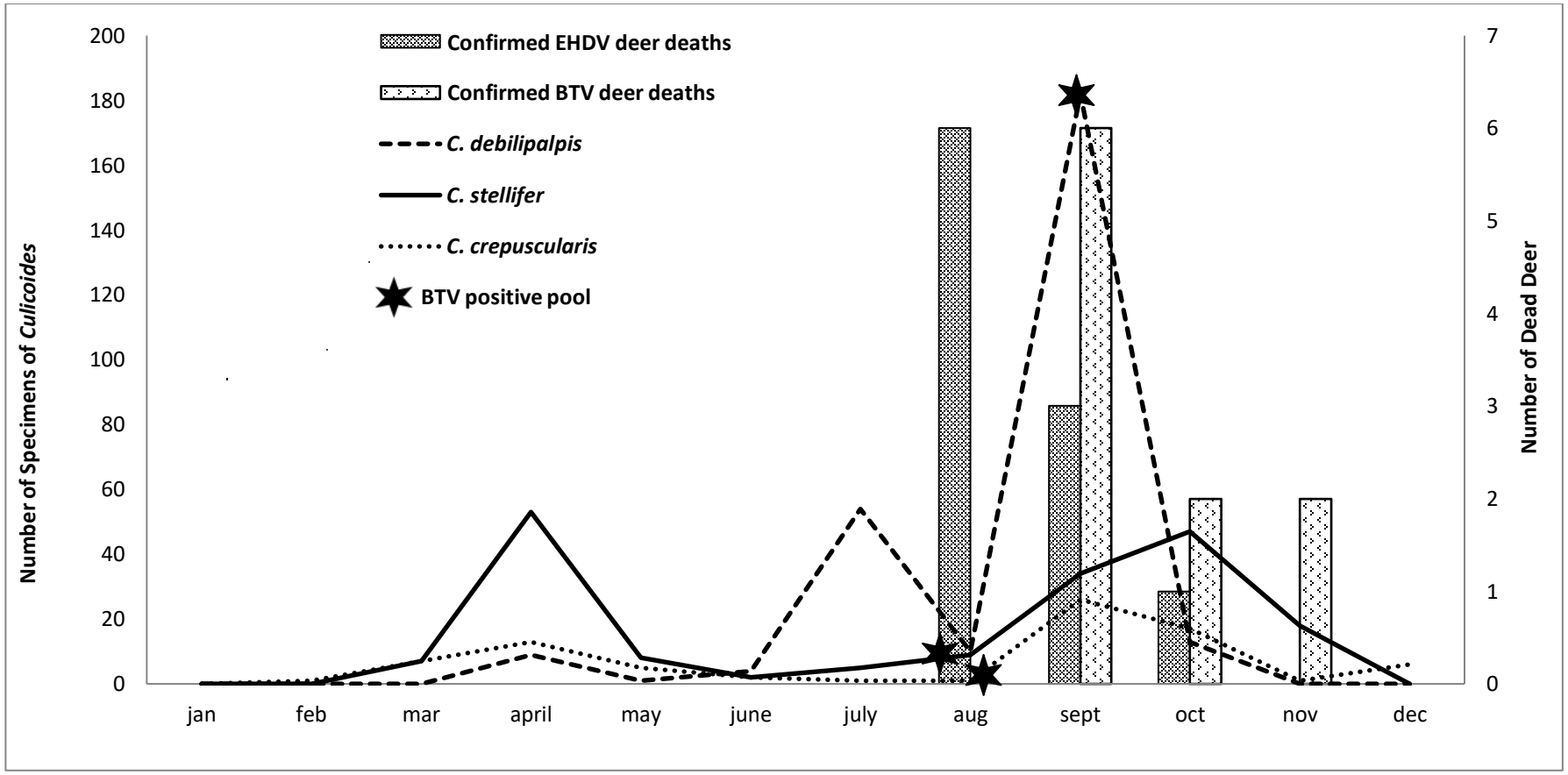

Figure 3.1. The 2012 seasonal incidence of three species of *Culicoides* which tested positive for bluetongue virus (BTV) by RT-qPCR versus the number of deer deaths that were confirmed BTV or EHDV by RT-qPCR from the Bob R. Jones Idlewild Research Station.

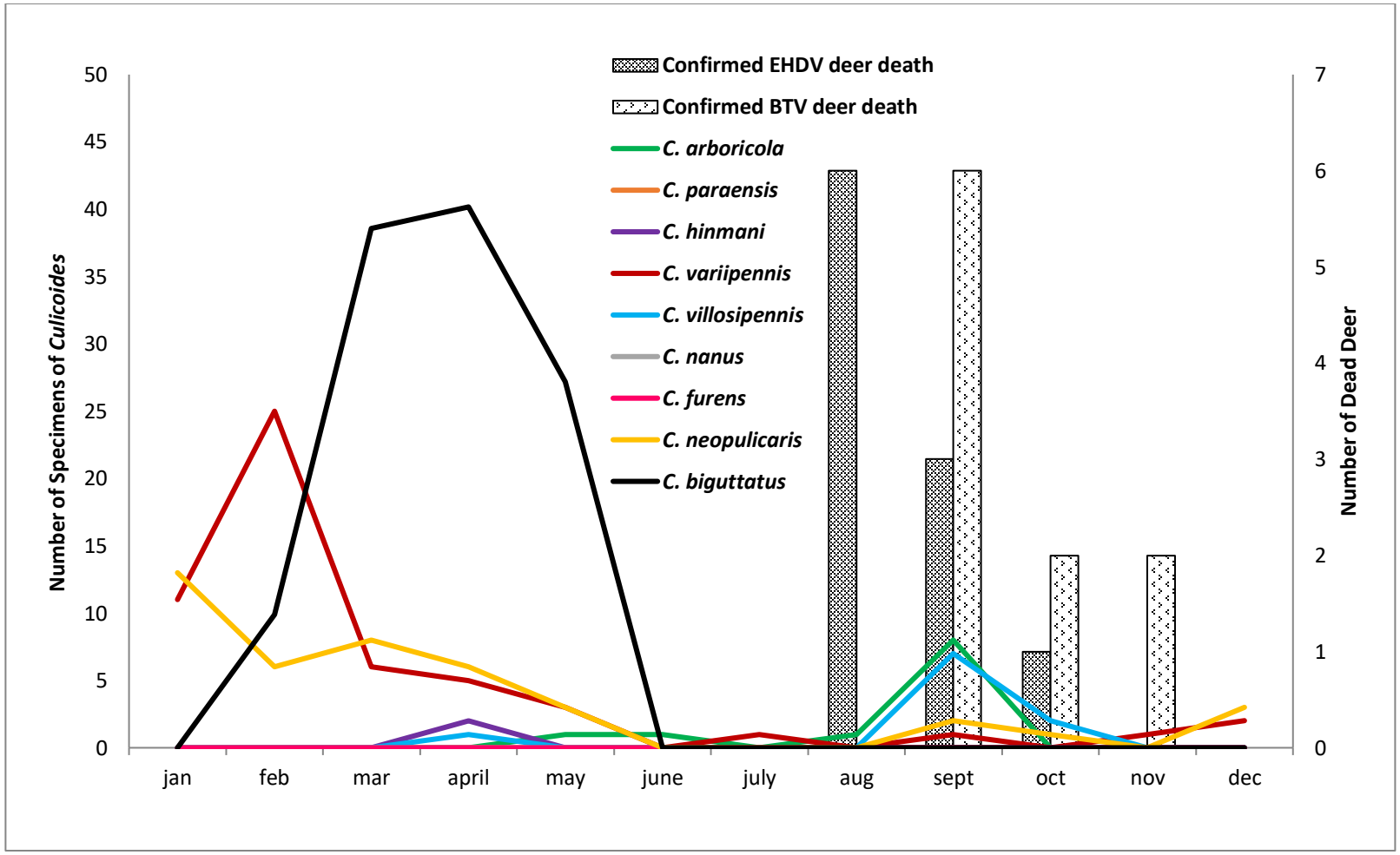

Figure 3.2. The 2012 seasonal incidence of eight species of *Culicoides* which are not implicated as vectors of BTV or EHDV and tested negative by RT-qPCR versus the number of deer deaths that were confirmed BTV or EHDV by RT-qPCR at the Bob R. Jones Idlewild Research Station

represented a predominant serotype in the U.S. with 50 isolations from 12 states. This serotype was the primary virus isolated from white-tailed deer in Louisiana, Florida, Arkansas, Michigan, and Wisconsin in 2012.

The cattle tested in this study had a much higher exposure rate than the deer. Out of 30 seronegative cows, 25 seroconverted for BTV or EHDV during the 2012 HD transmission season indicating an 80% exposure rate. One explanation for the lower exposure rate in deer (only 36%) could be that they were confined to one area while the cattle were free to move around in much larger areas and were rotated among pastures. Additionally, cattle may be more attractive hosts to the *Culicoides* midges due to their larger size and the amount of carbon dioxide they release in comparison to deer, or the host preference of the midge species can explain why cattle are more frequently exposed. Although cattle are normally asymptomatic to BTV and EHDV infections, it is important to note that they do develop high titers following exposure to these viruses and thus can serve as a source of infection (Bonneau et al. 2002).

Between 1996 and 2012, there was no recorded epizootic of HD at the BJIRS, but 12.5 % of the adult white-tailed deer were seropositive for BTV or EHDV prior to the 2012 vector season, potentially indicating BTV and EHDV exposure to deer without observed occurrence of clinical HD. During the 2012 transmission period, 18 deer seroconverted for BTV or EHDV, clearly indicating transmission of these viruses among the herd. Of the 25 adult deer that died from BTV and/or EHDV infection, 11 had known antibody status prior to the epizootic and only two of these were seropositive indicating previous exposure to the viruses. Therefore, we were not able to describe evidence of protective antibody for this study. However, 41 of 64 deer that were seronegative prior to the epizootic were still seronegative after, indicating a measured 36% exposure rate.

An annual exposure rate of this magnitude could lead to enzootic stability in both penned and wild white-tailed deer. For example, of the serum samples taken from hunter-killed deer from Camp Avondale in Clinton, La less than 8 km from the Idlewild Research Station, over 85% were seropositive for BTV or EHDV (Table 3.2). These results demonstrate that wild deer in Louisiana are frequently exposed to BTV and EHDV and may approach enzootic stability for certain serotypes. Similarly, Stallknecht et al. (1996) serologically tested 685 white-tailed deer in Texas and found 84% to be BTV or EHDV seropositive yet there was a lack of reported disease, indicating an enzootic stability.

In 2012, 18 of the 76 adult deer that died at the Idlewild Research Station were confirmed to have BTV or EHDV by RT-qPCR. These deer showed classic symptoms of HD such as weight loss, lethargy, decreased appetite, lameness, and hypersalivation before death, and necropsy findings, as well as RT-qPCR results, confirmed their death as a result of the disease. A total of 81 deer deaths were recorded at BJIRS in 2012, but samples could not be taken from every animal due to technical limitations. Therefore, the number of confirmed HD cases may be an underestimate of the magnitude of the outbreak. In the white-tailed deer herd there was a 50% mortality rate in the animals that were exposed to BTV or EHDV. Mortality rates due to EHDV and BTV in white-tailed deer can vary greatly depending on several factors including weather, vector abundance, serotype, host immunity, and viral dosage rates (Nettles and Stallknecht 1992). Mortality rates can reach 90% in BTV or EHDV infected deer herds in the wild and nearly 100% can be seropositive in some areas (Stallknecht et al. 1996). In Louisiana, approximately 200 deer deaths were reported in 2012, not including the deer from BJIRS (personal communication Jim LaCour). Considering the large number of deer die-offs reported in the mid-west and states well north of Louisiana in 2012, the most probable

explanation for the lower mortality rate observed in the state and for this study is a combination of enzootic stability and genetic selection in southern U.S. deer for lower susceptibility to HD (Gaydos et al. 2002b).

Natural transmission of EHDV and BTV is considered to be vector borne, but there have been reports that other modes of BTV and EHDV transmission may exist. There have been several studies with supporting evidence for oral transmission of EHDV among white-tailed deer. Ditchfield et al. (1964) isolated EHDV-2 from the feces of deer that were orally infected with EHDV-2. More recently, Gaydos et al. (2002a) observed contact transmission of EHDV-2 among white-tailed deer in the apparent absence of arthropods. Likewise, there are studies to show evidence of oral transmission of BTV in ruminants under certain conditions (Jochim et al. 1965, Menzies et al. 2008, Clarke et al. 2019). In the current study, we worked with confined deer and therefore carefully tracked the occurrence of cases among the different pens. There were no clusters of cases of a single virus or serotype that would indicate contagious exposure (Table 3.4) leading us to conclude that BTV and EHDV were likely transmitted by biological vectors and that oral transmission did not play a role in the epizootic.

Twenty-one cattle were brought to BJIRS in January of 2012; some of them were originally from different areas of Texas where BTV-12 and EHDV 6 have been previously documented. One of these naturally infected cattle was RT-qPCR positive for BTV-12 for 175 days; the rest of the tested cattle were RT-qPCR positive for shorter durations, which is consistent with other reports (Katz et. al 1994, Bonneau et. al 2002). Although BTV nucleic acids can be detected for up to 180 days using RT-qPCR techniques, cattle blood was shown to be infectious to *C. sonorensis* for only 21 days post infection using virus isolation (Singer et al. 2001, Bonneau et al. 2002). In the current

study, the one cow that remained BTV positive for 175 days likely did not have high enough virus titers to be infectious to an insect vector. Unfortunately, the exact Cq value was not reported for this animal, but given the maximum length of BTV/EHDV viremia in cattle is approximately 60 days (Singer et al. 2001), a transmission event to set up the outbreak in the deer herd would have to have occurred in May or June. The transferred cattle arrived in January, therefore two transmission events need to occur in order to bridge from winter to vector season, assuming cattle only remain infectious for 60 days. Since the transferred cattle were housed together, we cannot rule out contagious transmission as one of the two events. More research is needed to determine if BTV and EHDV can be persistent for long periods in the mammalian host. Since there was no clinical disease observed by the deer manager in the deer herd for the 15 years prior to 2012 and there had been no descriptions of BTV 12 or EHDV 6 in Louisiana prior to this study, we propose that it is plausible that the transferred cattle from Texas were the source of the outbreak.

We captured high numbers of specimens from three species of *Culicoides* (*C. debilipalpis*, *C. stellifer*, and *C. crepuscularis*) during an epizootic of BTV and EHDV among captive white-tailed deer (Figure 3.1). We also detected BTV viral RNA in three pools of midges from these three species captured in August and September, consistent with the time frame of the outbreak of deer deaths that were confirmed to be BTV. Previous studies have incriminated these 3 species as suspected vectors of BTV (Wieser-Schimpf et al. 1993, Becker et al. 2010, McGregor et al. 2018). However, many studies emphasize and focus on *C. sonorensis*, a known U.S. vector of BTV, while little is known about the biology, ecology, and vector competence of other *Culicoides* species. We did not detect any EHDV positive insect pools in this study. Similarly, Becker et al. (2010)

tested 204 pools of 1620 specimens from 10 different species of *Culicoides* captured in Louisiana using light traps and 5 pools were positive for BTV using RT-PCR but none were positive for EHDV. In the present study, it is significant that the first EHDV confirmed deer death occurred on August 8, which is one month earlier than the first BTV confirmed deer death (Figure 3.1). The evidence in this report supports the idea that the vector-transmission cycle for EHDV is different than that of BTV.

In this study, all midges were captured using CDC black-light traps baited with dry ice during an epizootic of BTV and EHDV among cattle and white-tailed deer. We did not catch any specimens of *C. sonorensis* in our light traps baited with CO_2, which is the midge trapping method used in several studies (Wieser-Schimpf et al. 1993, Hunt and Tabachnick 1996, Mayo et al. 2012). For this study, we were able to correlate peaks of vector activity with BTV and EHDV transmission because we had the opportunity to follow a herd of penned deer with acute infections and clinical symptoms. Most previous studies were based on seroconversion, rather than current infections, and were retrospective. For example, Stallknecht et al. (1995) tested serum from nearly 1400 white-tailed deer for the presence of BTV and EHDV antibodies using AGID tests and compared results from different regions of Georgia, but did not have the opportunity to collect tissues from dead deer during an epizootic. From the results of this study, we can infer that in the absence of *C. sonorensis* the most highly probable vectors in Louisiana for BTV among ruminants are *C. debilipalpis*, *C. crepuscularis*, and *C. stellifer.* In this study, none of the specimens of *Culicoides* captured were positive for EHDV. The insect trapping method used in this study may not have been optimal for vectors of EHDV which is an important subject to address for future studies.

CHAPTER 4. SEVEN YEAR PROSPECTIVE STUDY CHARACTERIZING BLUETONGUE AND EPIZOOTIC HEMORRHAGIC DISEASE VIRUS AMONG CULICOIDES AND WHITE-TAILED DEER

2.1 Introduction

Biting midges in the genus *Culicoides* are considered to be the primary biological vectors of bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV) worldwide. These viruses cause hemorrhagic disease (HD) in wild ruminants and bluetongue disease (BT) and epizootic hemorrhagic disease (EHD) in domestic livestock. Worldwide, 27 serotypes of BTV and 7 serotypes of EHDV exist, and there are different species of *Culicoides* midges that are considered to be the primary vectors of these orbiviruses within different regions (Gibbs et al. 1994, Tabachnick 1996, Anthony et al. 2009, Schulz et al. 2016). For example, the major vector of BTV in Africa is *Culicoides imicola*, but BTV has also been isolated from *C. tororoensis* and *C. milnei* (Walker and Davies 1971). The principal vectors of EHDV in Africa are members of the *C. schultzei* group and *C. brevitarsis* in Australia; however, in Central and South America, Japan and southeast Asia the vectors for EHDV are largely unknown (Mellor et al. 1984, Parsonson and Snowdon 1985).

In the U.S. the only 2 confirmed vectors of BTV are *C. sonorensis* and *C. insignis* since BTV was isolated from field caught specimens and transmission to sheep was demonstrated in the lab (Foster et al. 1963, Tanya et al. 1992); *C. sonorensis* is also the only accepted competent vector of EHDV (Holbrook et al. 2000) . However, many recent studies have implicated other midge species, such as *C. stellifer, C. debilipalpis,* and *C. venustus,* as vectors of BTV/EHDV especially in areas among the southeastern

U.S. where *C. sonorensis* is rare (Smith et al. 1996, Becker et al. 2010, McGregor et al. 2019).

Although all ruminant species are susceptible to BTV and EHDV, white-tailed deer (WTD, *Odocoileus virginianus*) and certain sheep breeds are the most vulnerable as mortality rates can be greater than 80% in some populations (Beringer et al. 2000). Both BTV and EHDV are enzootic in the southern U.S. and are normally transmitted in late summer into early fall and can cause significant economic losses especially in white-tailed deer herds (Rushton and Lyons 2015). Hemorrhagic disease is considered to be the most important infectious viral disease of white-tailed deer in the U.S. (Nettles et al.1992). Cattle are considered reservoir hosts for BTV and EHDV because infected animals are normally asymptomatic but can have high titers of the viruses (MacLachlan et al. 1994). Cattle are very significant in the movement of these viruses because they can harbor the virus for extended periods and they are often transported across large regions.

In the U. S., five bluetongue virus serotypes (2, 10, 11, 13, and 17) were known to be present prior to 1998, but 11 additional serotypes have been reported in the southeastern United States from white-tailed deer and cattle (Ostlund 2010, Maclachlan et al. 2013). Historically, EHDV-1 and 2 were considered to be endemic in the U.S. and recently EHDV-6 was isolated from white-tailed deer (Allison et al. 2010). There is concern of BTV and EHDV spreading northward in North America; a recent increase of reported HD in the Midwest and Northeast U.S. has been reported (Stallknecht et al. 2015). Furthermore, BTV was reported in 2015 and EHDV in 2017 from white-tailed deer in Ontario, Canada for the first time (Allen et al. 2019, Maclachlan et al. 2019).

Large scale epizootics of HD in WTD tend to occur every 8-10 years in the U.S., but sporadic smaller endemic outbreaks can occur every 2-3 years (Stallknecht and

Howerth 2004). The differences in these cycles may be explained by combined effects of herd immunity and fluctuations in vector populations. The most recent outbreak of HD in the U.S. occurred in 2012; over 25,000 deer deaths were reported for Michigan and Missouri while South Dakota Game, Fish and Parks gave refunds on hunting licenses due to the large number of white-tailed deer deaths (Flinn and Sumners 2013). Although the impact of HD on the reduction of wild deer populations was apparent in 2012, the vectors associated with this epizootic were not well described.

Most studies on the epidemiology of HD which include vector surveillance corresponding with disease occurrence have been conducted over short periods of 1-2 years. In this study, we monitored BTV/EHDV transmission within a captive deer herd over seven consecutive years and conducted vector surveillance as well. The objectives were to survey *Culicoides* midge populations, test for BTV and EHDV antibody serological changes in penned white-tailed deer, and use reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) assays to detect the presence of BTV and EHDV nucleic acid in midges as well as in tissues and whole blood collected from deer.

2.2 Materials and Methods

The study was conducted at the Louisiana State University Agricultural Center Bob R. Jones Idlewild Research Station (BJIRS) near Clinton, Louisiana (30.817954N, -90.97324W). A reproductive herd of approximately 50 adult crossbred beef cattle is maintained among six pastures ranging from 18 to 30 hectares. Approximately 100 captive white-tailed deer, also a reproductive herd, are maintained among 11 fenced enclosures, ranging from approximately 0.1 to 1.8 hectares. There is also a reproductive herd of roughly 30 red deer (*Cervus elaphus*) at the station. There is no buffer zone between the cattle pastures and deer pens, and one of the cattle pastures is separated from

the deer pens by less than 100 meters. The white-tailed deer herd is closed, although the 830 hectare facility is home to wild white-tailed deer; however, the cattle herd is not closed.

- Deer Death Records and PCR Diagnostics

From June 2012 through November 2018 the white-tailed deer herd was monitored daily and pen location, tag identification number, and death date were recorded for adult and fawn white-tailed deer. Whole blood was collected in 10mL purple top vacutainer tubes (reference number 366643; BD Vacutainer, Franklin Lakes, NJ) from symptomatic live deer, and tissues such as spleen and bone marrow were harvested from dead deer. Tissue homogenates were prepared by crushing 0.10-0.20g of tissue in a 2.0mL Eppendorf vial using a plastic pestle and a pellet pestle motor (model #749540). Then, 900 µL of media, or BA-1 buffer, consisting of Hanks M-199 salts, 0.05 M Tris, pH 7.6, 1% bovine serum albumin, 0.35g/L sodium bicarbonate, 100 U/mL penicillin, 100ug/mL streptomycin, and 1ug/mL fungizone was added to each vial. Whole blood was centrifuged at 3500rpm for 20 minutes and washed with phosphate-buffered saline (PBS) 3 times and then 5mL of BA-1 buffer was added to the tube. Viral RNA was isolated from the blood and tissue samples (100 µl blood sample or 100 µl tissue homogenate) using the Qiagen QIAmp viral RNA mini kit (catalog number 52906; Qiagen, Germantown, MD) using the manufacturer's instructions. BTV and EHDV multiplex reverse transcription and real-time PCR (RT-qPCR) utilized the Qiagen Rotor-Gene Multiplex RT-PCR kit (catalog number 204972; Qiagen, Germantown, MD). Master mix preparation and cycling conditions were as previously described (Becker et al. 2020b). Samples were considered EHDV positive when Cq values were less than 40 cycles and BTV positive when Cq values were 36 cycles or less.

Samples that were BTV or EHDV positive from RT-qPCR were serotyped using a gel-based reverse transcriptase polymerase chain reaction assay utilizing the Qiagen One Step RT-PCR kit (catalog number 210212; Qiagen, Germantown, MD). The primers used for BTV-10 and 12 and EHDV-1, 2 and 6 are listed in Table 4.1. The RNA template-primer mix, which consisted of 5uL of RNA template and 4uL of each forward and reverse primer, was denatured at 95°C for 5 minutes. The RT-PCR mixture for each reaction contained 5 µl RNase-free water, 5 µl 5X RT-PCR buffer, 1 µl 10 mM dNTP mix, and 1 µl enzyme mix. Denatured RNA template-primer mix (13uL) was mixed with 12 µl of the RT-PCR mixture and amplified using a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA). The RT-PCR cycling conditions were as follows: 50°C for 30 min, 95°C for 15 min, 40 cycles of amplification at 94°C for 45 sec, 56°C for 45 sec and 72°C for 1 min, with a final 10 min extension at 72°C, followed by refrigeration. Amplified PCR products (5uL) were stained with 2uL of SYBERsafe (Invitrogen), loaded onto 1.5% agarose gel, and ran at 110 volts for 45 minutes. Amplified DNA bands were visualized under UV light. Samples containing bands of the correct size were considered positive (422bp for BTV-3,585bp for BTV-10, 717bp for BTV-12, 603bp for BTV-14, 921bp for EHDV-1, 705bp for EHDV-2 and 564bp for EHDV-6).

Virus isolation from PCR positive samples was attempted utilizing baby hamster kidney cells (BHK) and Vero cells which are susceptible to *Orbivirus* infection (Wechsler and McHolland 1988) using the protocols described in Becker et al.(2020a).

- Serology

From September 2011 through September 2018, blood samples were obtained yearly in the winter using red top vacutainer tubes (reference number 367820; BD

Table 4.1. Primers for BTV and EHDV used in RT-PCR serotype assays for samples from deer and cattle at the Bob R. Jones Idlewild Research Station near Clinton, Louisiana from 2012-2018.

Primer	Sequence (5′–3′)	Amplicon size (bp)
BTV-3F	GCCGAGCGTAAATGTTGC	422
BTV-3R	GGCTCTTCCTCCATATTC	
BTV-10F	TTCCGCAACAGATGATGGG	585
BTV-10R	CGGGCATTAAACATCGGTG	
BTV-12F	AAGTGGGATGCGATCATGG	717
BTV-12R	CCTTCCGGGTAGCATATGTAG	
BTV-14F	GATGAGCTAGCGATCCCAAT	603
BTV-14R	CTCATCAGTTGTGGCTCTCT	
EHDV-1F	GCGTTGGACGTTGATGTG	921
EHDV-1R	GTCCCAGGGATCATCATTAC	
EHDV-2F	CGTGAACGCTTTAGAAGG	705
EHDV-2R	GATCTCGACCCGTCTTATC	
EHDV-6F	CTTGATCCCGTTGCCTTT	564
EHDV-6R	TCCAGGTGATCTCAGTCA	

Vacutainer, Franklin Lakes, NJ) from all penned white-tailed deer on the premises; after 24 hours at room temperature, serum was collected from the tubes by centrifuging at 3000 rpms for 10 min and stored at -20°C. All serum samples were screened for antibody to EHDV and BTV, separately, using the agar gel immunodiffusion (AGID) test following the manufacturer's recommended procedures. The AGID test kits for BTV and EHDV were obtained from Veterinary Diagnostic Technology, Inc., Wheat Ridge, CO.

- Insect Trap Study and PCR

From January 2012 through December 2018, miniature Centers for Disease Control (CDC) black light traps (model 512; John W. Hock Co., Gainesville, FL) baited with 2 kg of dry ice in igloo containers were hung approximately 1.5 m above ground at different sites greater than 50 m apart on certain days. Six-volt 20 amp hour rechargeable batteries were used to energize the traps. The traps were spread within a 1km radius among deer pens, cattle pastures, wooded areas, and near the edge of a freshwater lake. The traps were deployed before dusk and collection containers were retrieved no later than 2 hours after sunrise. Six to eight traps were used for each trapping event and the black light was removed from half of the traps in 2016- 2018.

For each year, weekly trappings were conducted from June-November and traps were deployed once or twice for all other months. There was a total of 1326 trap nights during the entire 7 year study. Captured insects were kept on a cold chain and stored in a cooler with frozen blue ice until arriving in the lab where they were transferred to a refrigerator at 4º C. Subsequently, the insects were sorted using a dissecting microscope and a chill table (BioQuip®, Gardena, CA). Ceratopogonids were sorted into genus by examining the wing venation, number of antennal segments, and maxillary palps using the *Manual of Neartic Diptera* (1981) as a reference. Members of the genus *Culicoides* were sorted by species through examination of wing patterns using the keys of (Blanton and Wirth 1979) as a reference; voucher specimens were confirmed by mounting, dissecting and clearing followed by examination of the spermathecae.

Field-collected midges from the genus *Culicoides* were pooled by species, site, and date and placed into Eppendorf 2.0mL Safe-Lock tubes (Cat. No 022363352). Pools of at least 5 up to a maximum of 50 midges were homogenized manually with a plastic pestle using the procedure described above. Then 900 uL BA-1 buffer was added and the

sample was vortexed for 30 seconds. Viral RNA was extracted and BTV/EHDV multiplex reverse transcription and real-time PCR (RT-qPCR) was performed on all pools of midges using the protocols described above.

Blood fed specimens of *C. debilipalpis* were collected from a baffle trap (Foil et al. 1984) using a mouth aspirator that used a live calf that had a natural infection of BTV as bait. The specimens were transferred to paper cups with fine mesh screen across the top and the methods described in Erram et al. (2018) were used for potential oviposition on wet filter paper. The eggs were collected and transferred to 1.5mL Eppendorf vials and RNA was extracted and RT-qPCR for BTV and EHDV was performed on 5 pools of 20-30 eggs using the protocols described in Becker et al. (2020b).

4.3 Results

- Deer Death Records and PCR Diagnostics

From 2012 to 2018 there were a total of 112 white-tailed deer deaths at the BJIRS that were confirmed for BTV or EHDV using RT-qPCR on tissue samples collected at necropsy (Table 4.2). The deer died in late summer and early fall (August- November) each year and showed classical symptoms of HD before death. The Cq values for BTV ranged from 16.0 -34.5 and for EHDV from 17.4-39.2. The serotype results over 7 years revealed BTV-10, BTV-12, EHDV-1, EHDV-2, and EHDV-6 in the deer tissues. In 2012, there were 8 confirmed cases of EHDV and 6 for BTV (Figure 4.1). One deer was positive for EHDV-1, 7 for EHDV-6, and 6 for BTV-12. In 2013 there were 3 confirmed cases of BTV-12 and 2 for BTV-10 and no EHDV detected (Figure 4.2). In 2014 there were 42 confirmed cases of BTV-12 but again no EHDV (Figure 4.3). In 2015, tissues from 3 white-tailed deer were positive for EHDV-2 and 10 for BTV-12 (Figure 4.4). In 2016, there was only 1 deer that was positive for EHDV-6 and 8 were positive for BTV-

Table 4.2. Number of confirmed RT-qPCR BTV and EHDV deer deaths and serotype detected by RT-PCR in white-tailed deer from August-November in 2012-2018 at the Bob R. Jones Idlewild Research Station near Clinton, La.

Year	BTV-10	BTV-12	EHDV-1	EHDV-2	EHDV-6
2012	0	6	1	0	7
2013	2	3	0	0	0
2014	0	42	0	0	0
2015	0	10	0	3	0
2016	0	8	0	0	3
2017	0	4	3	0	13
2018	0	9	0	0	0

12 (Figure 4.5). In 2017, there were 3 positive for EHDV-1, 13 for EHDV-6, and 4 for BTV-12 (Figure 4.6). In 2018, there were 9 confirmed cases of BTV-12 and no EHDV was detected in deer tissues (Figure 4.7). In a previous study, Becker et al. (2020b) reported EHDV-2, EHDV-6, and BTV-12 from cattle blood collected from the herd at the station. Virus isolation from the cattle blood was successful for BTV-12 on BHK and Vero cells, but not for either strain of EHDV.

- Serology

Data for BTV and EHDV antibody were combined for analysis since there can be cross reactivity when using AGID tests (Stallknecht 1991). In 2012 prior to the vector season, only 15% of the deer herd was seropositive for BTV or EHDV antibodies (Table 4.3). During the transmission season, 17 out of 64 deer seroconverted for either BTV or EHDV and 43% of the herd was seropositive post transmission season. In 2013, 11 out of 54 deer seroconverted for BTV or EHDV and in 2014, 19 out of 57 seroconverted. At the end of 2015, 67% of the herd was seropositive for BTV or EHDV. In 2016, 17 out of 52 deer seroconverted for BTV or EHDV and 14 out of 49 seroconverted in 2017. At the end of 2018, 78% of the deer herd was seropositive for BTV or EHDV (Table 4.3).

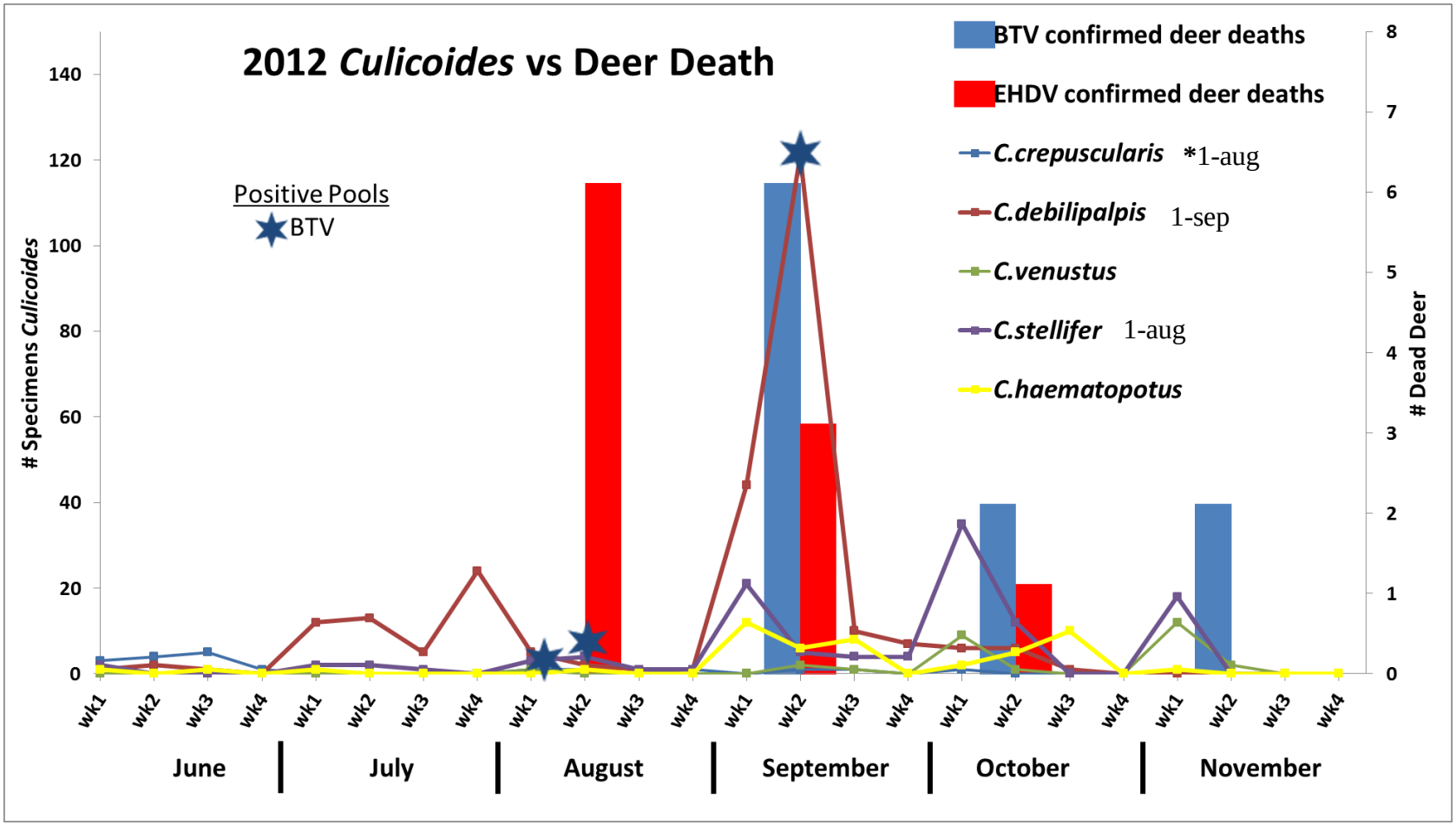

Figure 4.1. Number of specimens from 5 *Culicoides* species which are suspected vectors of BTV/EHDV captured per week in CDC light traps versus the number of BTV confirmed deer deaths in 2012 at the Bob R. Jones Research Station near Clinton,La.

*number of positive pools per month

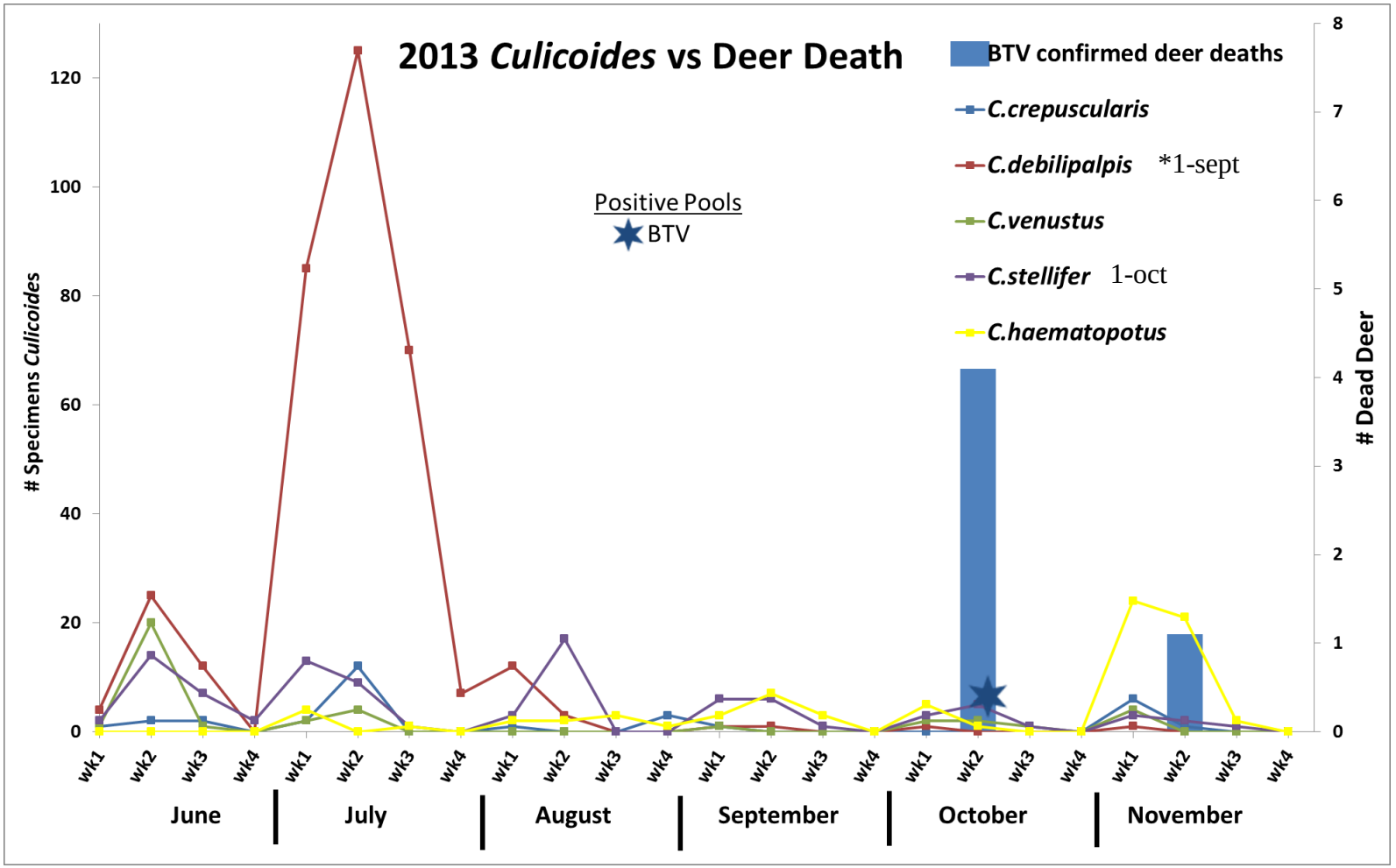

Figure 4.2. Number of specimens from 5 *Culicoides* species which are suspected vectors of BTV/EHDV captured per week in CDC light traps versus the number of BTV confirmed deer deaths in 2013 at the Bob R. Jones Research Station near Clinton, La.

*number of positive pools per month

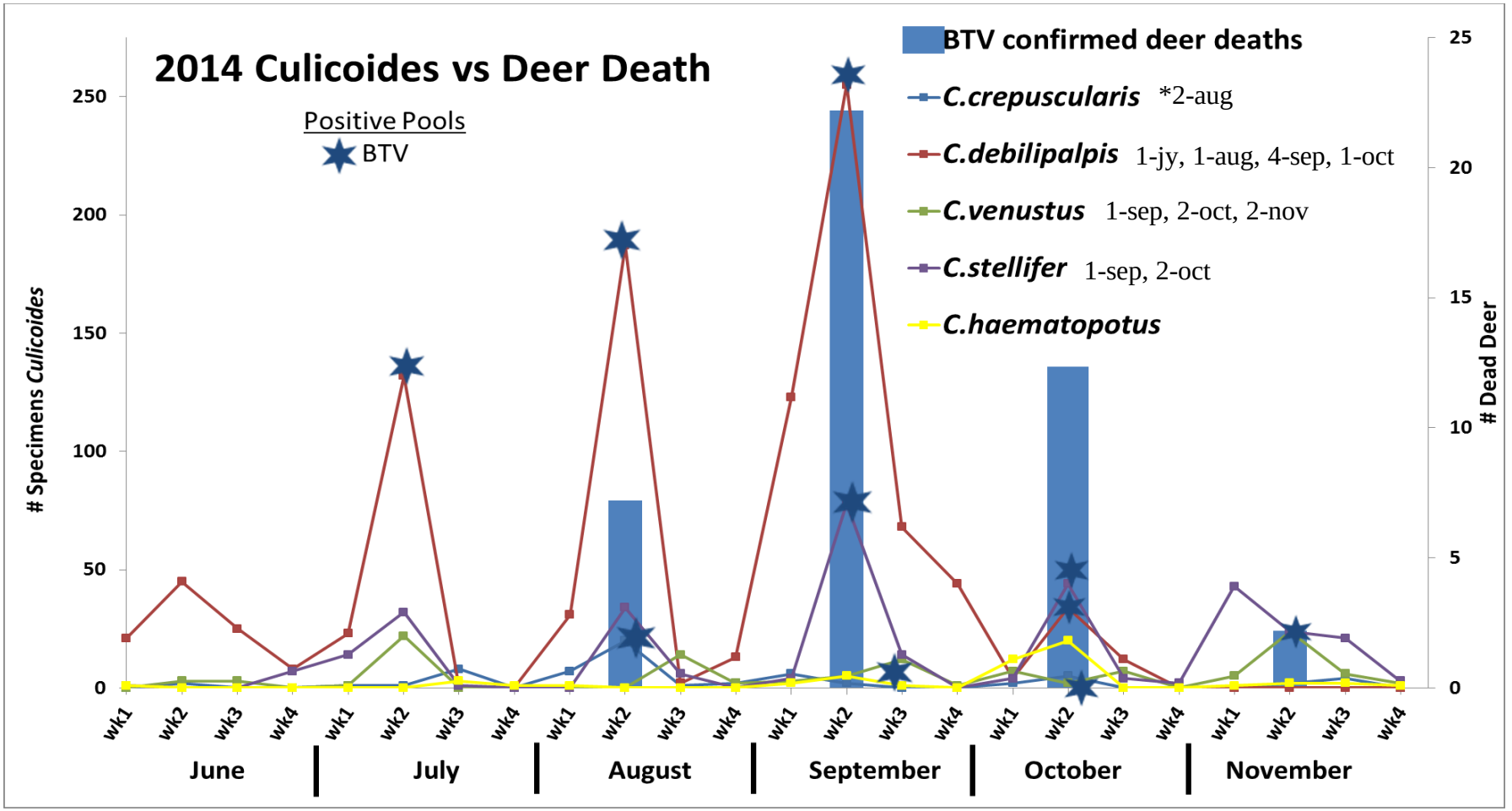

Figure 4.3. Number of specimens from 5 *Culicoides* species which are suspected vectors of BTV/EHDV captured per week in CDC light traps versus the number of BTV confirmed deer deaths in 2014 at the Bob R. Jones Research Station near Clinton,La.

*number of positive pools per month

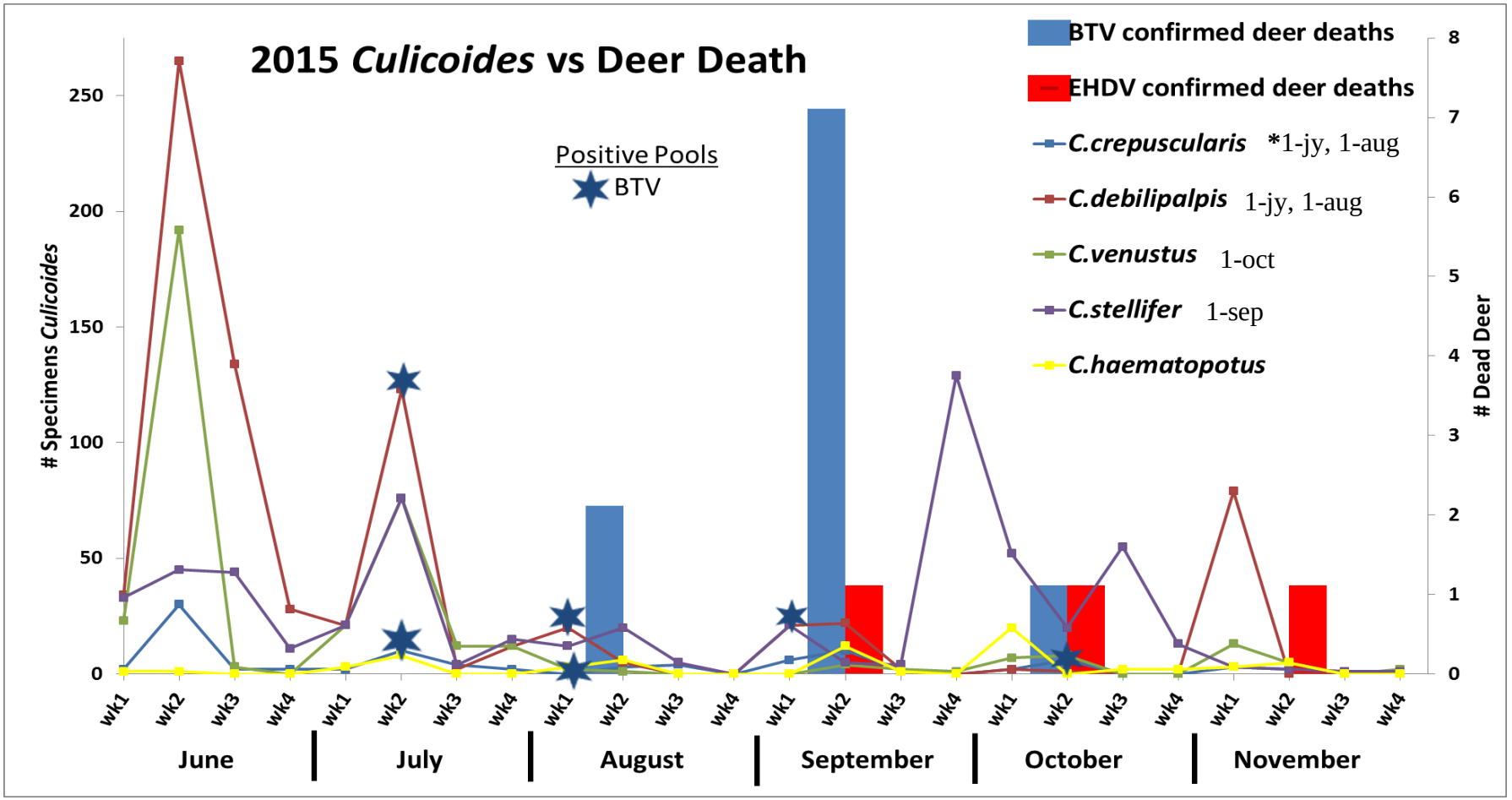

Figure 4.4. Number of specimens from 5 *Culicoides* species which are suspected vectors of BTV/EHDV captured per week in CDC light traps versus the number of BTV and EHDV confirmed deer deaths in 2015 at the Bob R. Jones Research Station near Clinton, La.

*number of positive pools per month

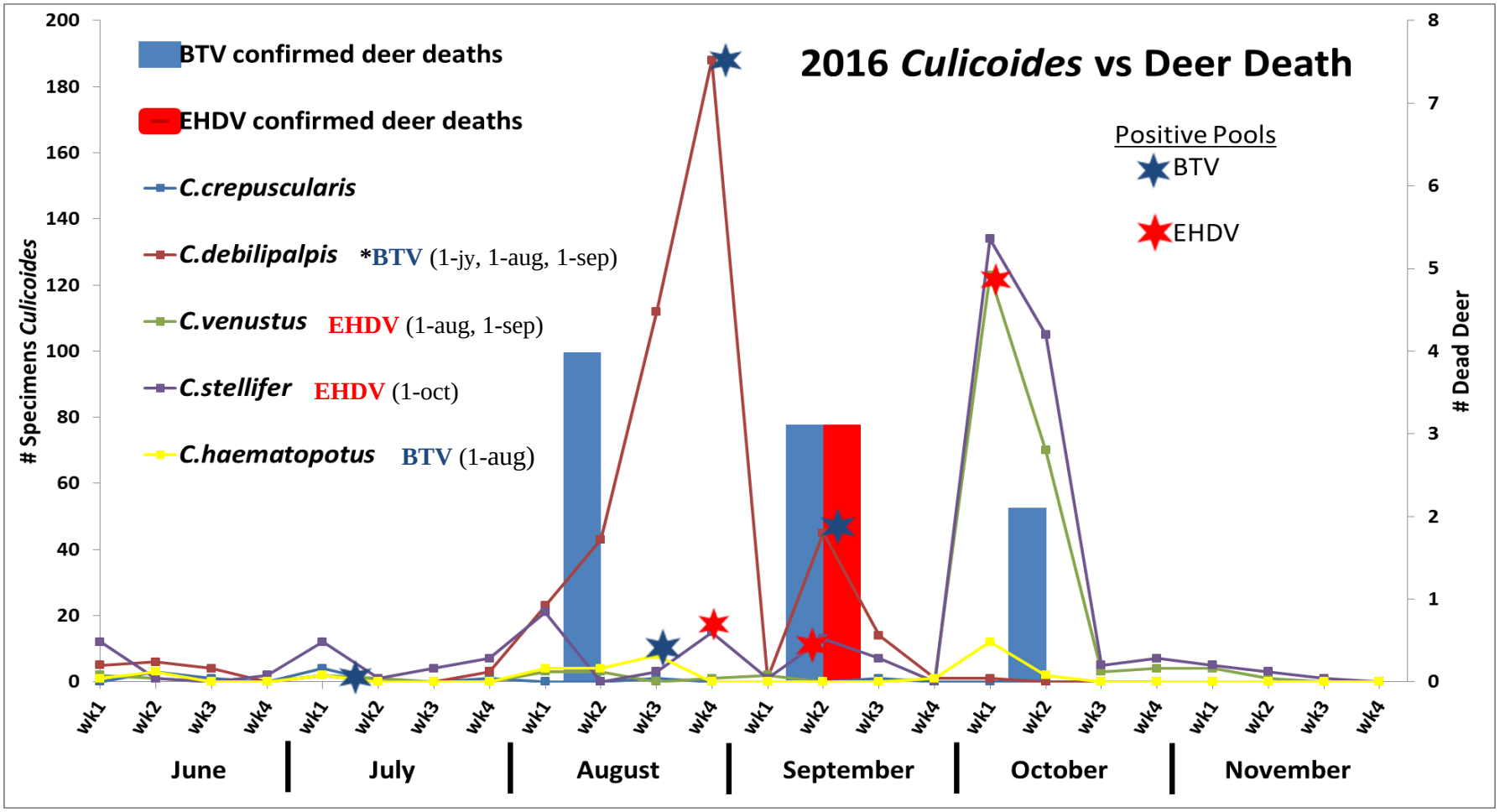

Figure 4.5. Number of specimens from 5 *Culicoides* species which are suspected vectors of BTV/EHDV captured per week in CDC light traps versus the number of BTV and EHDV confirmed deer deaths in 2016 at the Bob R. Jones Research Station near Clinton, La.

*number of positive pools per month

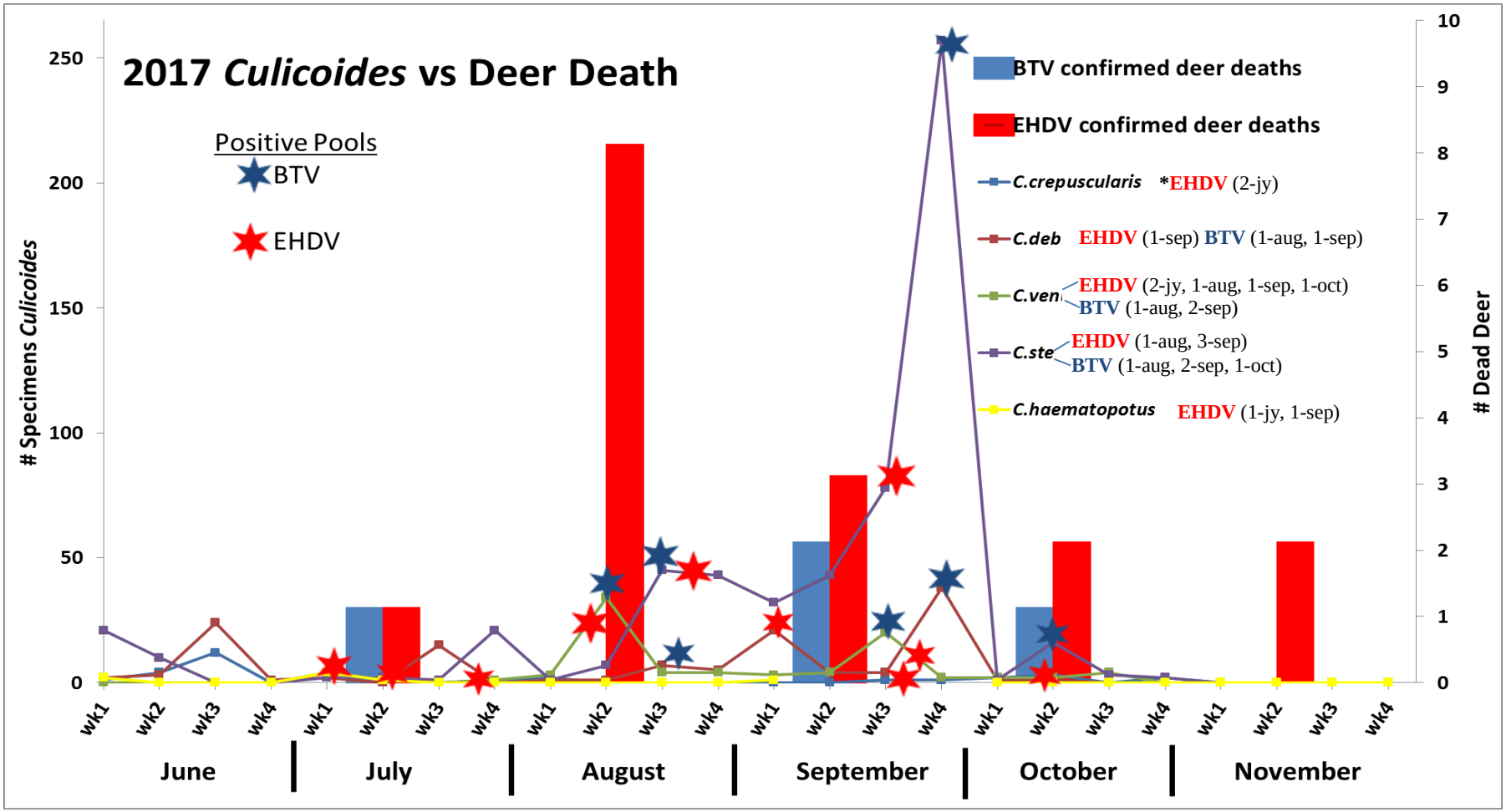

Figure 4.6. Number of specimens from 5 *Culicoides* species which are suspected vectors of BTV/EHDV captured per week in CDC light traps versus the number of BTV and EHDV confirmed deer deaths in 2017 at the Bob R. Jones Research Station near Clinton, La.

*number of positive pools per month, *C. deb=C. debilipalpis, C. ven=C. venustus*

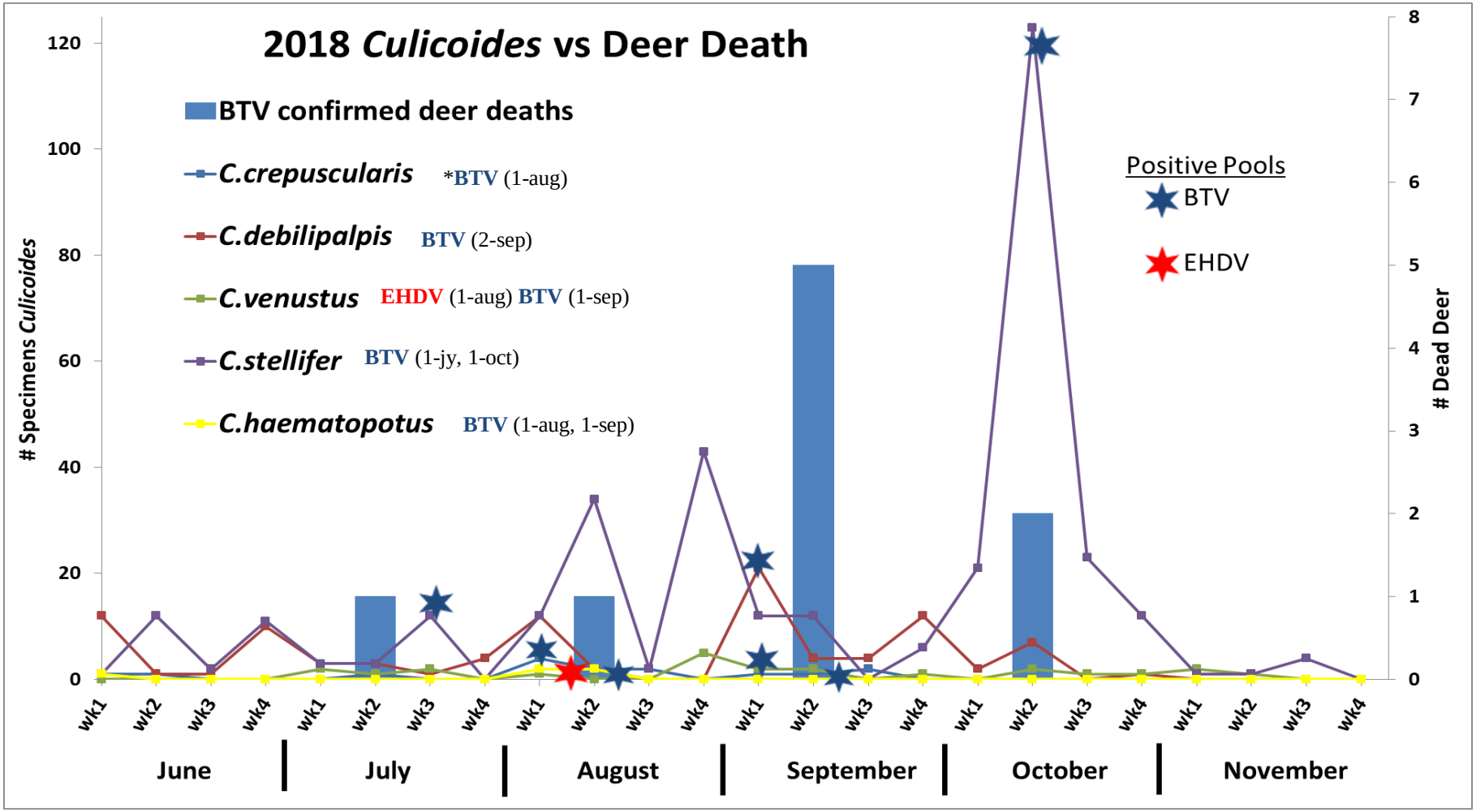

Figure 4.7. Number of specimens from 5 *Culicoides* species which are suspected vectors of BTV/EHDV captured in CDC light traps per week versus the number of BTVand EHDV confirmed deer deaths in 2018 at the Bob R. Jones Research Station near Clinton, La.

*number of positive pools per month

Table 4.3. Percent of adult white-tailed deer herd seropositve by agar gel immunodiffusion (AGID) test for BTV/EHDV pre and post vector season, total number of dead deer, number of deer seropositive and seronegative preseason that died from BTV/EHDV infection, and the number of deer that seroconverted for BTV/EHDV and survived from 2012-2018 at the Bob R. Jones Idlewild Research Station near Clinton, La.

Year	Pre % sero + BTV/EHDV	Total # Dead	# Dead		#seroconverted BTV/EHDV and survived	Post % sero + BTV/EHDV
			Pre Sero +	Pre Sero -		
2012	15	81	2	9	17 of 64	43
2013	43	5	1	4	11 of 54	45
2014	45	42	6	10	19 of 57	51
2015	51	13	3	10	16 of 59	67
2016	67	11	4	7	17 of 52	74
2017	74	28	6	22	14 of 49	76
2018	76	11	6	3	15 of 44	78

- Insect trap study and PCR

A total of 24,859 specimens in 1711 pools representing the following 15 species of *Culicoides* were captured from January 2012 through December 2018 and tested for the presence of BTV and EHDV using RT-qPCR; *C. arboricola* Root and Hoffman, *C. biguttatus* Coquillet, *C. crepuscularis* Malloch, *C. debilipalpis* Lutz, *C. furens* Poey , *C. haematopotus* Malloch, *C. hinmani* Khalaf, *C. nanus* Root and Hoffman , *C. neopulicaris* Wirth, *C. paraensis* Goeldi, *C. pusillus* Lutz,*C. stellifer* Coquillet,*C. variipennis* Coquillett, *C. villosipennis* Root and Hoffman, and *C. venustus* Hoffman (Table 4.4). Specimens from 46 pools from 5 species (*C. crepuscularis*, *C. debilipalpis*, *C. haematopotus*, *C. stellifer*, and *C. venustus*) tested positive for BTV and 18 pools were positive for EHDV during the study (Table 4.5). For BTV, *C. debilipalpis* had the highest number of positive pools (15) and for EHDV *C. venustus* had the most positive pools (7). Specimens from these 5 species accounted for over 60% of insects captured throughout the study and over 90% in the months of June-October 2012-2018, which is the primary EHDV/BTV transmission season. In every year from 2012-2018, specimens from 1 to 5 species were positive for BTV (Figures 4.1-4.7). However, the first PCR positive pools for EHDV came from 2016; 2 for *C. stellifer* and 1 for *C. venustus* (Figure 5). In 2017, specimens from all 5 species were PCR positive for EHDV among 14 pools; *C. debilipalpis* (1), *C. stellifer* (4), *C. haematopotus* (2), *C. crepuscularis* (2), and *C. venustus* (5) (Figure 4.6). In 2018, the only EHDV positive pool was from *C. venustus* (Figure 7). The overall MIR of the midge pools for BTV ranged from 3.45 (*C. stellifer*) to 13.51 (*C. crepuscularis*) and for EHDV ranged from 0.29 (*C. debilipalpis*) to 5.42 (*C. venustus*) (Table 4.4). Out of 10 pools of *C. debilipalpis* eggs, 2 were RT-qPCR positive

Table 4.4. Number of pools, specimens, BTV/EHDV positive pools and minimum infection rate (MIR) for 15 species of *Culicoides* captured in CDC light traps from 2012-2018 at the Bob R. Jones Idlewild Research Station near Clinton, La.

Species	#pools	#specimens	#BTV+ pools	BTV MIR	#EHDV+ pools	EHDV MIR
C. arboricola	82	193	0	N/A	0	N/A
C. biguttatus	326	12784	0	N/A	0	N/A
C. crepuscularis	102	456	6	13.51	2	4.50
C. debilipalpis	317	3531	17	4.85	1	0.29
C. furens	3	8	0	N/A	0	N/A
C. haematopotus	48	384	3	7.39	2	4.93
C. hinmani	2	8	0	N/A	0	N/A
C. nanus	17	41	0	N/A	0	N/A
C. neopulicaris	21	253	0	N/A	0	N/A
C. paraensis	3	5	0	N/A	0	N/A
C. pusillus	13	42	0	N/A	0	N/A
C. stellifer	491	3543	12	3.45	6	1.73
C. variipennis	126	2268	0	N/A	0	N/A
C. villosipennis	13	26	0	N/A	0	N/A
C. venustus	147	1317	10	7.75	7	5.42

Table 4.5**.** Total number of pools and specimens from all captured *Culcoides* midges from 2012-2018 at the Bob R. Jones Idlewild Research Station near Clinton, La and number of BTV or EHDV positive pools for 5 species of *Culicoides* captured in CDC light traps.

				Number of Positive Pools				
Year	**#pools**	**#specimens**	**Virus**	***C. crepuscularis***	***C. debilipalpis***	***C. haematopotus***	***C. stellifer***	***C. venustus***
2012	174	1334	BTV	1	1	0	1	0
			EHDV	0	0	0	0	0
2013	251	1809	BTV	0	1	0	1	0
			EHDV	0	0	0	0	0
2014	308	8049	BTV	2	7	0	3	5
			EHDV	0	0	0	0	0
2015	362	5394	BTV	2	2	0	1	1
			EHDV	0	0	0	0	0
2016	226	5206	BTV	0	3	1	0	0
			EHDV	0	0	0	2	1
2017	188	1987	BTV	0	2	0	4	3
			EHDV	2	1	2	4	5
2018	202	1080	BTV	1	2	2	2	1
			EHDV	0	0	0	0	1

for BTV. Virus isolation attempts for BTV and EHDV were unsuccessful for all pools of midges and eggs.

4.4 Discussion

In 2012, we confirmed EHDV-1, EHDV-6 and BTV-12 in tissue samples from white-tailed deer that died at this study site (Becker et al. 2020b). This was the first report of EHDV-6 and BTV-12 in Louisiana. The results of the 2012 study are presented in this report in a different format than previously published to provide a reference point for the 7-year prospective study described in this manuscript. Allison et al. (2010) provided the first account of EHDV-6 in the U.S. in isolates from white-tailed deer in Indiana and Illinois, and determined that the isolates were from a reassortmant of an endemic EHDV-2 and an exotic EHDV-6 (Allison et al. 2012). The first isolation of BTV-12 in the U.S. came from white-tailed deer samples in Texas (Ostlund 2010), and Becker et al. (2020b) subsequently reported BTV-12 for the first time in Louisiana. In addition to detecting EHDV-6 and BTV-12 in white-tailed deer for the first time in La., we also demonstrated infection of these two viruses in cattle imported from Texas where these strains had been shown to occur in the spring prior to the epizootic in the white-tailed deer herd (Becker 2020b). Notably over the next 6 years, BTV-12 accounted for 97% of the deaths in deer associated with BTV and EHDV-6 accounted for 83% of deaths associated with EHDV.

In 1999, the largest U.S. outbreak of HD caused by EHDV-1 since 1975 occurred from Georgia up into New Jersey where over 100 counties in 10 states reported white-tailed deer die offs or chronic lesions (Murphy et al. 2006). Since then outbreaks caused by EHDV-1 in white-tailed deer have not been observed especially in the southern U.S. Similarly, EHDV-6 did not cause any major outbreaks of HD since it was first detected in

2006 until 2012, when it was reported in 14 states in the southeastern and Midwest U.S. (Ruder et al. 2017). For EHDV-2, outbreaks in 2007 and 2012 occurred when over 250 and 125 isolates were made from white-tailed deer, respectively (Ruder et al. 2017). In this 7 year study, the overall death toll for white-tailed deer confirmed EHDV positive was 28 with most deaths due to EHDV in August each year, and the yearly EHDV serotype detection was variable in deer (Figure 4.8). For example, in 2012 we reported EHDV-1 and EHDV-6 in the deer herd, but for 2013 and 2014 no EHDV was detected (Table 4.2). In 2015, there were only 3 confirmed EHDV-2 deer and no other serotypes of EHDV. In 2016, there was only one deer that died of EHDV and it was EHDV-6. Then EHDV-1 and EHDV-6 were found in deer samples in 2017 but in 2018 there was no EHDV in the deer herd. Previous studies indicate that it is not uncommon to detect serotype diversity for EHDV from year to year in livestock ruminants (Homan et al. 1990, Stallknecht et al. 1995). For example, (Ruder et al. 2017) reported EHDV-6 in only 2 out of 10 years in white-tailed deer in Louisiana. The exact reason or mechanism for this sporadic yearly occurrence of EHDV serotypes is of interest and infected wild deer may have been the source of the EHDV infection in those years.

Studies have shown that certain BTV strains can be persistently transmitted in herds of ruminants over several years. For example, Stott et al. (1985) detected BTV-13 and 17 in cattle every year for over a 3 year period; additionally, Stallknecht et al. (1995) followed a white-tailed deer herd from 1989-1991 and detected BTV-13 every year. Over the seven year period of this study, the total number of deer deaths confirmed for BTV-12 was 82 with most occurring in September of each year (Figure 4.8). In 2012, which was the largest outbreak of HD to have been observed at the BIJRS, tissues from 6 deer were confirmed BTV-12 positive. In addition, 11 of 12 cattle tested in November

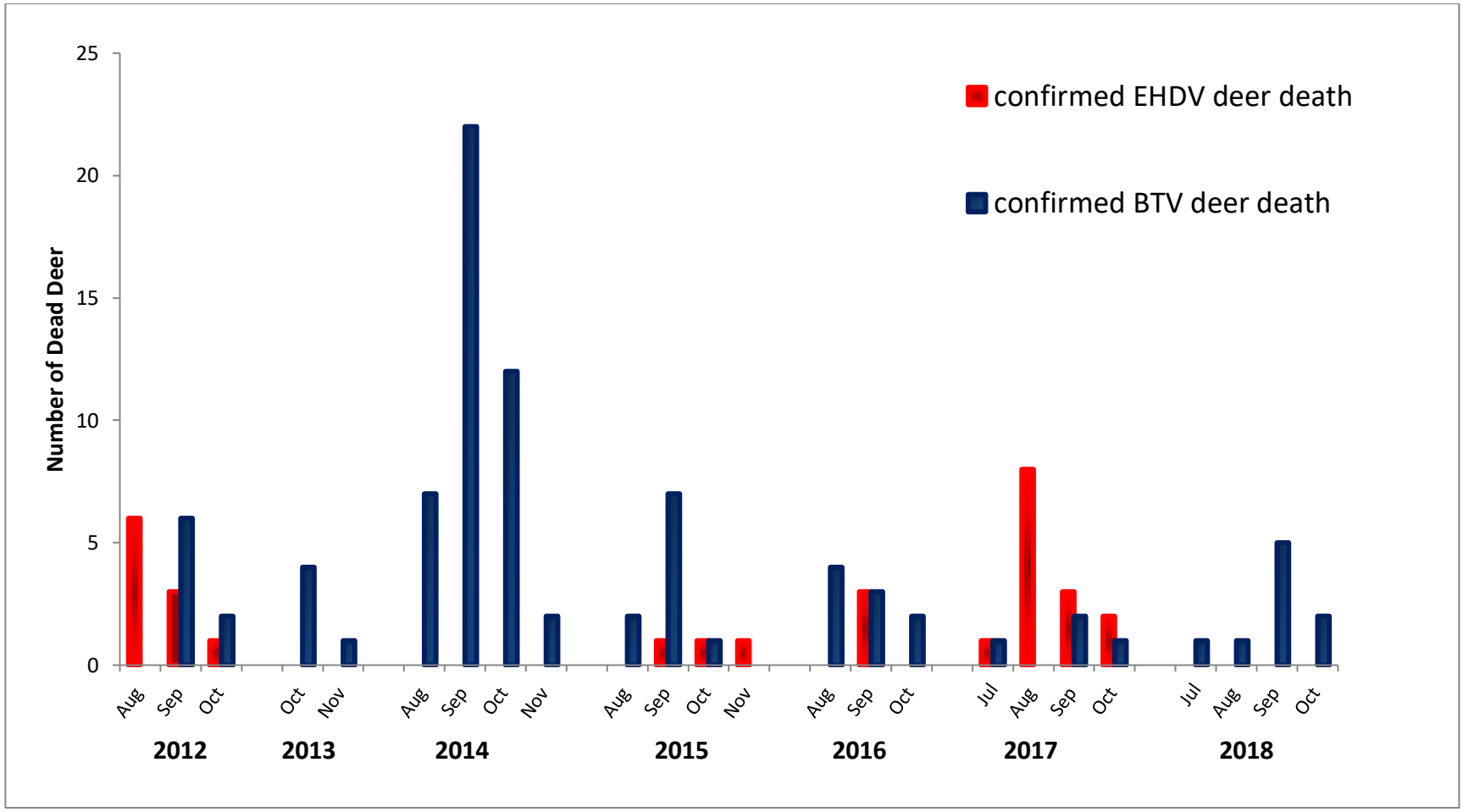

Figure 4.8. Number of BTV and EHDV confirmed white-tailed deer deaths from 2012-2018 at the Bob R. Jones Idlewild Research Station near Clinton, La.

2012 were BTV-12 positive (Becker et al. 2020b). In Europe, BTV-8 was first detected in 2006 when only 2000 farms were affected; BTV-8 overwintered and in 2007 and 2008 caused one of the largest outbreaks of BTV in European history affecting sheep, cattle, and goats on over 60,000 farms with an economic impact totaling over 200 million U.S. dollars (Wilson and Mellor 2008). Our results were similar to the BTV-8 outbreak in Europe in that BTV-12 overwintered and we recorded transmission in all 7 subsequent years and experienced several outbreaks of BTV-12 after the initial introduction. Since BTV persisted in both studies, it is more probable that BTV-12 is maintained at the station without the influence of wild WTD.

In the fall of 2011, only 12.5% of the white-tailed deer at the BJIRS were seropositive for BTV or EHDV. In 2012, 81 white-tailed deer died during vector season and over 70% of the fawn crop was lost after undergoing the largest BTV/EHDV outbreak observed at the station. Tissue samples were not taken from every deer that died in 2012 due to manpower restraints and loss of tissues due to scavengers, particularly for fawns, and therefore the number of confirmed cases for BTV/EHDV was underestimated. In 2012, only 2 of the 21 deer that died of BTV or EHDV were seropositive for BTV/EHDV before the outbreak. Therefore, we did not have enough data to conclude whether deer that were seropositive for BTV or EHDV before the 2012 HD transmission season were protected from BT or EHD. From 2013 to 2018, we determined serological status of the entire deer herd prior to and after vector season, and there was an overall increase for the percentage of BTV and EHDV seropositive deer after vector season each year throughout the study (Table 4.3). The percent mortality for seropositive adult deer remained 10% or less over the entire study with survival of at least 90 % of the adult deer that were seropositive before the transmission season in each year (Figure 4.9).

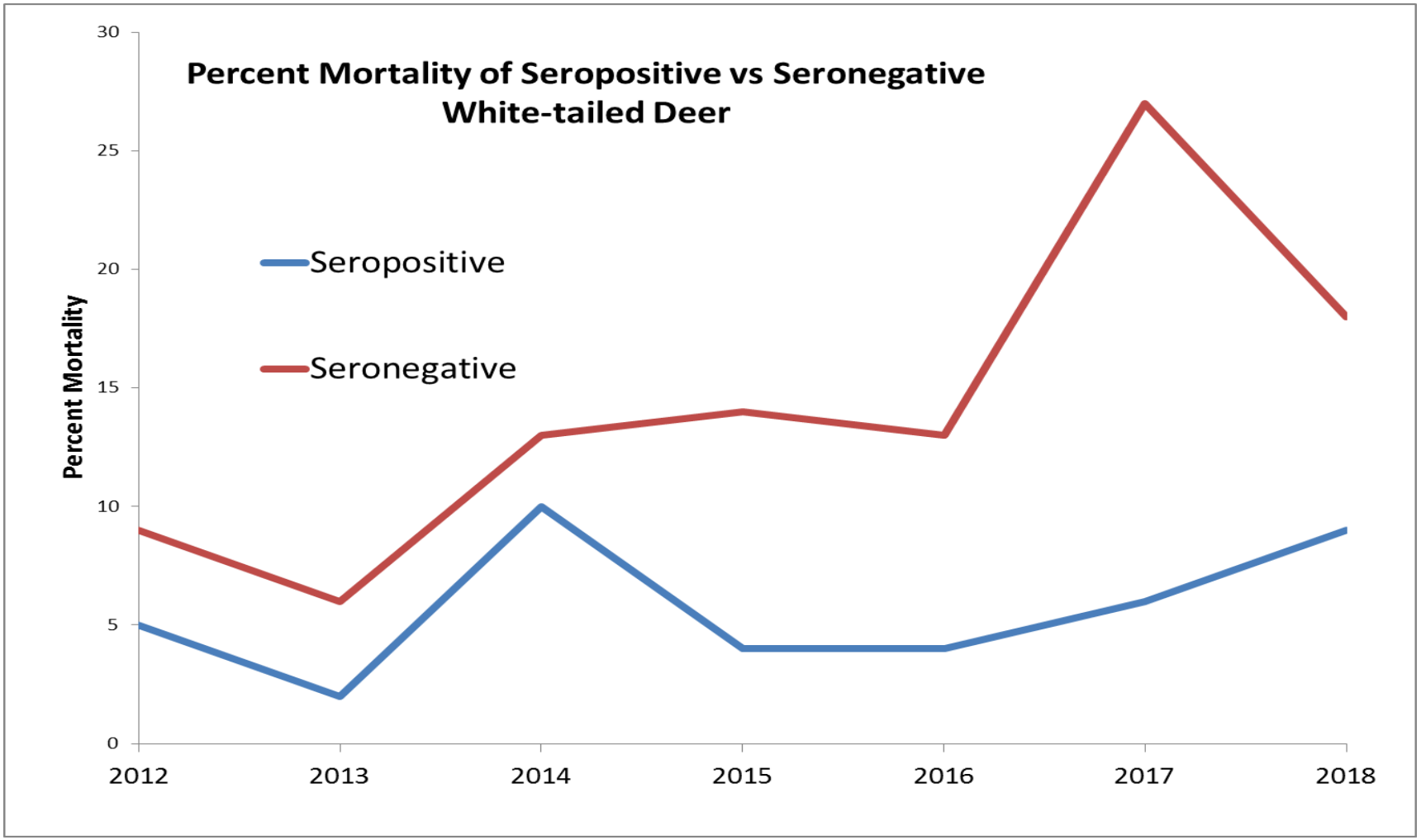

Figure 4.9. The percent mortality of BTV/EHDV seropositive versus seronegative adult white-tailed deer measured pre vector season using agar gel immunodiffusion test (AGID) from 2012-2018 at the Bob R. Jones Idlewild Research Station near Clinton, La.

The AGID test only detects precipitating antibodies and does not specifically test for protective immunity, but the results of these tests are often used to describe trends. The overall mortality rates in the adult white-tailed deer herd for seropositive deer was 5.9% compared to 23% for seronegative deer. Previous literature states that mortality rates can be as high as 90% in deer herds, but in our study the highest yearly mortality rate observed was 23% and herd immunity indicated the establishment of enzootic stability. Further studies would be required to validate the specificity of the herd immunity and the implications for wild deer populations following introduction of a new virus serotype such as BTV-12 and EHDV-6 were in 2012.

The 10 species captured that were not found to be PCR positive for BTV or EHDV (*C. arboricola, C. biguttatus, C. furens, C. hinmani, C. nanus, C. neopulicaris, C. paraensis, C. pusillus, C. variipennis C. villosipennis*) are consistent with previous literature with the exception of the following 3 species. Although no field collected positive pools have been observed in this study or others, specimens of *C. bigutattus* are abundant in areas of apparent BTV/EHDV transmission (Smith et al. 1996, Zhang 2017). Becker et al. (2020b) reported specimens from *C. biguttatus* were highly abundant in 2012 but only in the spring (March-April) prior to any transmission and that pattern persisted throughout the following 6 years. There have been previous studies incriminating 2 species (*C. paraensis* and *C. furens*) in the south as potential orbivirus vectors. Mullen et al. (1985) concluded that specimens of *C. paraensis* should be given attention as potential vectors of BTV or EHDV in the U.S. after collecting them from white-tailed deer although they did not find any PCR positive specimens. Becker et al. (2010) found one BTV positive pool of *C. furens* in south Louisiana in an area with BTV transmission among cattle and deer. Although *C. paraensis* and *C. furens* are not

confirmed vectors of BTV or EHDV, their potential role in the transmission of orbiviruses should not be discounted. Furthermore, specimens from the two confirmed vectors for BTV (*C. insignis* and *C. sonorensis*) and one EHDV vector (*C. sonorensis*) were not collected in this study.

Over the 7 year study, we found 72 PCR positive pools (54 for BTV, 18 for EHDV) from specimens of 5 species of *Culicoides* (*C. crepuscularis, C. debilipalpis, C. stellifer, C. haematopotus,* and *C. venustus*). These 5 species of *Culicoides* have been implicated previously as vectors for BTV and EHDV. In two separate studies in Louisiana, field captured specimens from *C. debilipalpis, C. crepuscularis, C. haematopotus,* and *C. stellifer* in areas of BTV/EHDV transmission were found to be PCR positive for BTV (Becker et al. 2020b, Becker et al. 2010). The current study marks the first record in Louisiana for field collected *C. venustus* found to be PCR positive for BTV. In one study, female specimens of *C. venustus* were fed BTV spiked blood and although the infection rate as very low, the authors did conclude that *C. venustus* became infected with BTV (Jones et al. 1983). For EHDV, *C. venustus* and *C. stellifer* have been recently implicated as vectors of EHDV in Florida after finding PCR positive pools from field collected specimens at deer farms (McGregor et al. 2019). Smith et al. (1996) reported that EHDV-2 can replicate in *C. debilipalpis* in the laboratory setting after feeding on viremic white-tailed deer. However, this is the first time field collected midges from *C. debilipalpis* have been found to be PCR positive for EHDV. This was also the case for the collected specimens of *C. crepuscularis* and *C. haematopotus* found to be PCR positive for EHDV; both species have been suspect vectors of EHDV because of their presence in areas of transmission, but no studies have found field caught PCR positive pools. Four of the 5 species positive for BTV/EHDV in this study, with the

exception of *C. debilipalpis*, have been collected with light traps as far north as London, Ontario, Canada in an area where white-tailed deer deaths were confirmed EHDV-2 (Allen et al. 2019).

In this study, specimens of *C. debilipalpis* were the only species that had BTV positive pools every year over the 7 year period for a total of 18 positive pools (Table 4.5). We also observed BTV-12 transmission in the deer herd each year and the abundance of *C. debilipalpis* specimens correlated with the confirmed BTV deer deaths especially when combining the data for all years (Figure 4.10). There was an observed abundance of this species in years 2012, 2014, and 2016 during the time frame when peak confirmed deaths from BTV or EHDV occurred (Figures 4.1, 4.3, and 4.5). The geographic distribution for U.S. is the southeastern states from Maryland and Kentucky to Florida and Louisiana (Blanton and Wirth 1979). Specimens of *C. debilipalpis* have been reported to feed on white-tailed deer in large numbers in the southern United States. For example, in Georgia (Smith et al. 1996) caught over 20,000 specimens of *C. debilipalpis* from a caged white-tailed deer in one morning. This species is considered to be a tree hole breeder; larvae of *C. debilipalpis* were reared from wet tree and stump holes in Florida and in Argentina large numbers of larvae were collected from tree holes of *Salix* spp. (Smith 1965, Ronderos et al. 2010). Mullen et al. (1985) showed that specimens of *C. debilipalpis* that were fed BTV-spiked blood can harbor the virus through replication and are capable of transmitting BTV to a host. Furthermore, Becker et al. (2010) reported 2 BTV-positive pools of *C. debilipalpis* from field-collected specimens in an area of hemorrhagic disease transmission in Louisiana. In this study, we found BTV positive pools of eggs deposited by female *C. debilipalpis*. Considering the

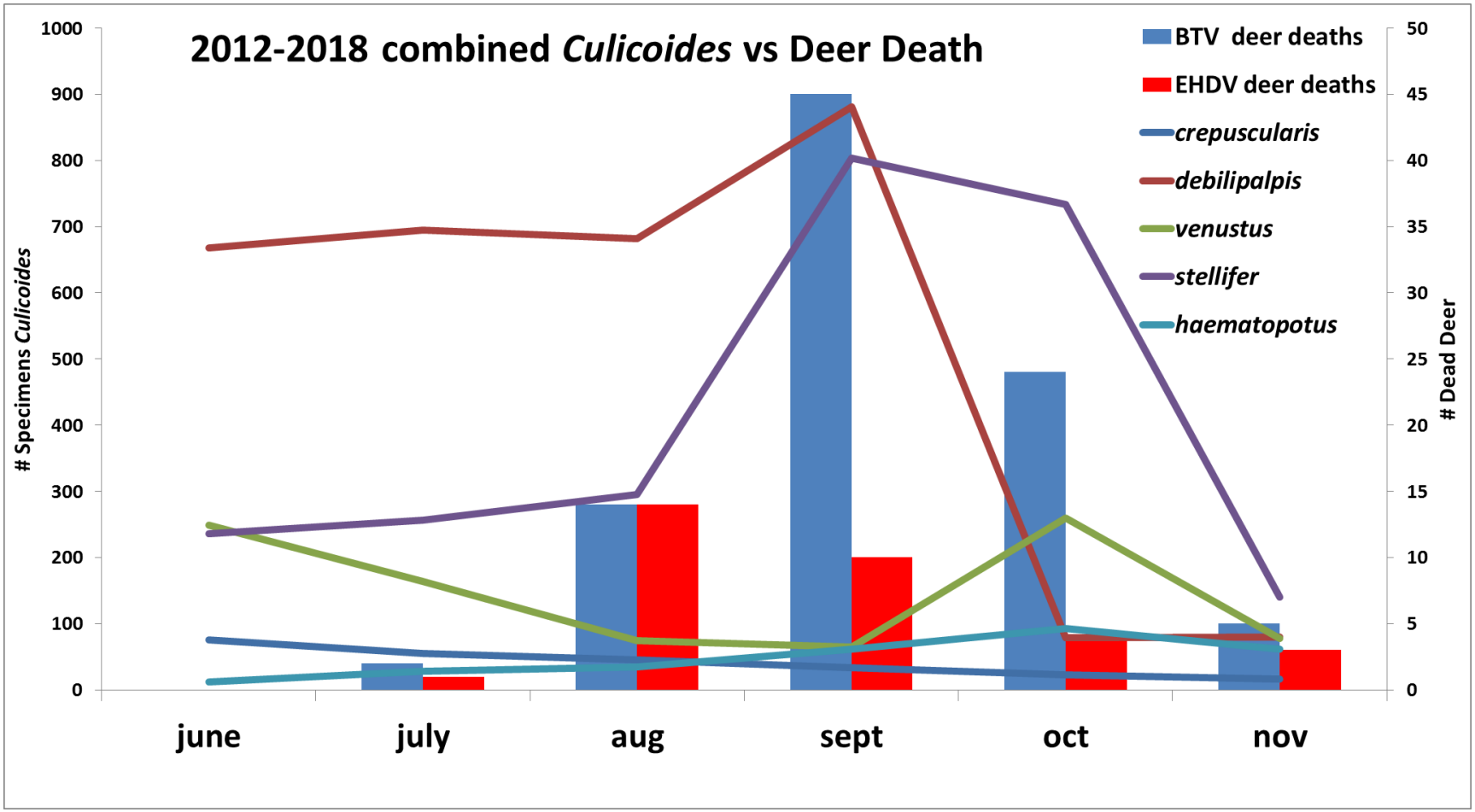

Figure 4.10. Number of specimens from 5 *Culicoides* species which are suspected vectors of BTV/EHDV captured per month in CDC light traps versus the number of BTV and EHDV confirmed deer deaths from 2012-2018 at the Bob R. Jones Research Station near Clinton, La.

evidence provided in previous studies and the information from this study, *C. debilipalpis* should be considered the primary BTV midge vector in Louisiana and probably most of the Southeast, and it is possible that BTV is vertically transmitted in this species. Specimens of this species may also play a role in EHDV transmission; we found one EHDV positive pool and studies have suggested this species as a putative vector for EHDV in the southeastern U.S. (Erram and Burkett-Cadena 2018).

Females of *C. crepuscularis* previously were considered to be mainly ornithophilic and had been recorded feeding on chickens (Hoffman 1925). However, more recent studies have reported specimens feeding on humans, cattle and sheep (Pickard and Snow 1955; Raich et al. 1997). In one study, this species was the most abundant in the Louisiana marsh, where BTV-1 was reported accounting for over 95% of the total specimens of *Culicoides* captured (Becker et al. 2010). The larvae of *C. crepuscularis* have been collected on the edge of streams and ponds in substrates moderate to high in organic matter (Battle and Turner 1972). In northern Colorado, White et al. (2005) found BTV genome segments 7 and 3 using PCR in larvae of *C. crepuscularis* collected in cattle pastures, but they did not detect all segments or culture BTV from larvae. Becker et al. (2010) reported one PCR positive pool of field collected specimens from *C. crepuscularis* in Louisiana and suggested this species to be a suspected BTV vector. Another study found field collected adult female specimens of *C. crepuscularis* to be PCR positive for EHDV-6 in Texas (Schoenthal 2015). The highest BTV MIR (13.5) of all species was from pools of *C. crepuscularis* in this study and the second highest (4.5) for EHDV MIR (Table 4.4). Therefore, considerable evidence supports the concept that this species may play a role not only in BTV transmission but EHDV as well.

In the current study, we found 12 pools of *C. stellifer* PCR positive for BTV and 6 pools positive for EHDV and observed a positive correlation in *C. stellifer* midge abundance versus confirmed deer deaths, particularly in years 2014-2018 (Figures 4.3-4.7). *Culicoides stellifer* is one of the most widespread species in North America and has been found in every state in the U.S. except Michigan, Washington, and Oregon; the larvae have been found in a variety of habitats with substrates enriched with organic materials (Blanton and Wirth 1979). However, the immature stages and habitats for most species of *Culicoides* species are understudied and not well characterized. In a recent oviposition study on *C. stellifer*, authors concluded that specimens preferred to oviposit on mud and vegetation from a known larval habitat over field water or deer manure (Erram and Burkett-Cadena 2018). Mullen et al. (1985) showed that specimens of *C. stellifer* were positive for BTV 7 days after intrathoracic inoculation. Becker et al. 2020b reported a BTV positive pool from *C. stellifer* collected during an epizootic of bluetongue and epizootic hemorrhagic disease among white-tailed deer. This species also has been implicated as a vector for EHDV; McGregor et al. (2019) reported 6 EHDV positive pools from field collected specimens in an area with EHDV transmission in white-tailed deer and correlated peaks of midge abundance with deer mortality. The evidence from this study shows that specimens of *C. stellifer* should be considered potential vectors for BTV and EHDV in Louisiana and possible much of North America within its range.

We found 3 BTV positive pools and 2 EHDV positive pools of *C. haematopotus* in this study. Specimens of *C. haematopotus* have been captured from cattle and aspirated from white-tailed deer in an area where BTV and EHDV were enzootic (Smith et al. 1996, Mullen et al. 1999). Larvae from this species have been found on the edges of ponds and streams that are not heavily polluted and Jones (1961) claimed this

species breeds in freshwater soil habitats. The range of *C. haematopotus* is throughout North America from northern Mexico up to southern Canada (Blanton and Wirth 1979). Becker et al. (2010) also found a BTV positive pool from this species from field collected specimens in an area with BTV transmission in Louisiana; therefore this species may play a role in BTV/EHDV transmission, at least in La.

In this study we found 15 BTV positive pools for *C. venustus* with a MIR of 7.75; although specimens from this species were not highly abundant, the midges captured had a high chance of being infected with BTV. This species was most abundant in October, especially in 2016, which indicates it may be more of a late season species capable of transmission when other species are less abundant (Figure 4.5). We found 7 EHDV positive pools for this species; specimens of *C. venustus* have been aspirated directly from cattle and are known to feed on mammals (Schmidtmann et al. 1980). The larvae from this species have been found in wet pastures breeding in muddy footprints of livestock and on stream margins polluted with hog feces (Blanton and Wirth 1979). The distribution of this species is Eastern North America from Wisconsin to Nova Scotia south to Louisiana and Florida. Numerous studies reported *C. venustus* present in areas of BTV and EHDV transmission among white-tailed deer (Tanya et al. 1992, Smith et al. 1996, McGregor et al. 2019). This species has been implicated as vector for EHDV by McGregor et al. (2019) who reported 16 positive pools from field collected specimens at a deer farm with confirmed EHDV infections. Our findings support the results from that study and confirm *C. venustus* is a probable vector for EHDV as well as BTV.

We conducted a unique multiyear prospective study on orbivirus transmission in which tissues from captive white-tailed deer that died of symptoms of HD were tested for BTV and EHDV in real-time and at the same time *Culicoides* midges were collected

using light traps and also tested for BTV and EHDV. Previous studies on orbivirus transmission were retrospective or followed only a small group of animals for a short period of time, usually 2 years or less.

There are 4 criteria that must be met to incriminate an arthropod and confirm as a vector of a pathogen, which include recovery of virus from non-blood fed field caught specimens and demonstration of the ability to become infected after feeding on an infected host or blood spiked with the pathogen, the ability to transmit the pathogen to a host by bite, and the abundance and association of field collections from the insects with the appropriate susceptible vertebrate host (Ruder et al. 2015). The 5 species collected in this study that were PCR positive for BTV and EHDV were also closely associated with white-tailed deer and in some years the peaks of collected midges occurred simultaneously with the confirmed BTV/EHDV white-tailed deer deaths and therefore met 2 of the criteria listed above. Previous studies have reported that *C. debilipalpis* midges prefer to feed on, and specimens have been collected directly off, white-tailed deer (Smith 1996, Mullen 1985). Although no BTV or EHDV was isolated from the collected midges, the fact that they were RT-qPCR positive is highly supportive of BTV/EHDV vector incrimination. One possible reason we were unsuccessful at growing BTV/EHDV from the midge pools could be that not all segments of the virus were present in the midges. White et al. (2005) showed that without viral protein (VP-2) or low levels of this segment in field collected Culicoides, recovering virus in cell culture can be difficult, especially in mammalian cell lines because VP2 is used in cellular attachment.

In some years the midge vector activity peaked at the same time as the confirmed deer deaths (Figures 4.1,4. 3, 4.5-4.7) and in other years the peaks of activity were not

obviously associated with deer deaths (Figures 4.2 and 4.4). The overall pattern of midge activity and deer deaths showed clear association of the suspected vector species with confirmed BTV and EHDV deaths (Figure 4.8). Therefore, it is important to conduct these types of studies over multiple years to get the overall concept of midge activity versus transmission. Observed peaks of competent vector midge activity do not always correspond with BTV/EHDV transmission patterns. Midge activity detected by trapping can differ nightly depending on abiotic factors such as moon phase, temperature, cloud cover, or wind even if midges are not finding and feeding on hosts during the night. By combining all the data from the midges and dead deer over 7 years, we get a clear understanding of the positive relationship of midge abundance versus confirmed deer death on a temporal scale (Figure 4.8). The evidence in this study supports the idea that the 2 major vectors driving BTV transmission in Louisiana are *C. debilipalpis* and *C. stellifer*. The other 3 species, *C. crepuscularis, C. haematopotus,* and *C. venustus,* also play an important role in orbivirus transmission. Our results confirm that *C. venustus* and *C. stellifer* can be competent vectors for EHDV as concluded by McGregor et al. (2018). Future studies are needed to support these species as competent vectors and elevate their status as confirmed BTV/EHDV vectors. The primary need for these important future studies is the establishment of laboratory colonies of suspected species, which allowed the important vector competence studies for *C. sonorensis* and were possible due to significant investment from the federal government.

CHAPTER 5. POSTMORTEM DETECTION OF BLUETONGUE AND EPIZOOTIC HEMORRHAGIC DISEASE VIRUSES IN THE BONE MARROW OF WHITE-TAILED DEER (ODOCOILEUS VIRGINIANUS)

5.1 Introduction

Bluetongue Virus (BTV) and Epizootic Hemorrhagic Disease Virus (EHDV) are vector-borne *Orbivruses* that negatively affect ruminants worldwide and cause significant economic losses to the agricultural industry (Tabachnick 1996). These viruses replicate in and are transmitted by biting midges of the genus *Culicoides*, which obtain the viruses from viremic ruminants (Mellor et al. 2000). Infections from BTV/EHDV can induce extreme morbidity in certain breeds of sheep and white-tailed deer (*Odocoileus virginianus*) with mortality rates exceeding 80% in white-tailed deer populations in some instances (Beringer et al. 2000). However, BTV and EHDV infections rarely manifest clinical signs in cattle, which are considered reservoir hosts for these two pathogens (MacLachlan 1994).

Hemorrhagic disease (HD) associated with BTV and EHDV infections is considered to be the most important infectious viral disease of wild deer in the U.S. (Nettles et al. 1992). In 2012, an epizootic of hemorrhagic disease occurred in white-tailed deer populations across the Midwestern United States totaling more than 25,000 dead. The 2012 HD epizootic had a significant negative economic impact through reduced sales and/or refunded hunting licenses (Hovey 2012; Stevens et al. 2015). In addition to wild populations, white-tailed deer farms, which contributed $3 billion USD to the economy and 29,000 jobs in 2007 (Anderson et al. 2007), often experience severe epizootics of hemorrhagic disease that lead to significant mortalities which can cause significant economic consequences from lost revenue in commercially managed herds.

HD is most commonly observed in late summer to early fall and two common symptoms of BTV or EHDV infection in white-tailed deer are high fever and dehydration, which may lead to deer seeking available water sources (Stallknecht and Howerth 2004). Therefore, an acute increase in deer mortalities around August-September combined with an increased number of deer carcasses found in or around water sources leads wildlife professionals to suggest that these mortalities were possibly due to EHDV or BTV. Molecular analysis of fresh tissue samples has been the primary mechanism to associate death with BTV/EHDV; however, fresh tissues are sometimes unavailable when deer carcasses are found in nature.

Orbiviruses are members of the family Reoviridae, which are non-enveloped double stranded RNA viruses that have a dual layer of capsid proteins. The atomic core of BTV is comprised of approximately 1000 self-assembled protein components, and this assembly yields extreme stability in the environment (Grimes et al. 1998) and can survive for several years in blood stored at 20°C. Blood rich organs, such as spleen, heart and lung are the tissues of choice to collect during necropsy for detection of BTV and EHDV, but these tissues often are scavenged or degrade prior to discovery of carcasses by wildlife professionals. Previous reports have indicated that BTV and EHDV are detectable in bone marrow (Brodie et al.1998, Afshar 1994, Stott et al. 1982). Bone marrow is naturally protected from many conditions that would render other tissues untestable fairly quickly. Protected by bone and a high fat content, marrow is resistant to desiccation, autolysis and most forms of scavenging. These innate properties could enable detection of certain viral particles possible for lengthy time post-mortem. If BTV and EHDV can be detected from white-tailed deer carcasses for extended periods of time post-mortem by molecular techniques, this could provide a useful tool for wildlife

biologists, agents, herd managers and deer farmers to help retrospectively determine a possible cause of mortality among white-tailed deer.

5.2 Materials and Methods

- Experimental Design

Tissues were collected from white-tailed deer maintained at the LSU AgCenter Bob R. Jones Idlewild Research Station (BJIRS) located in Clinton, Louisiana. The station maintains a reproductive herd of approximately 100 captive white-tailed deer among 11 fenced enclosures ranging from approximately 0.1 to 1.8 hectares, as well as reproductive herds of around 50 crossbred beef cattle (*Bos taurus*) and 30 red deer (*Cervus elaphus*). The white-tailed deer are monitored daily and a spleen and bone marrow sample are routinely collected from deer carcasses within 24 hours of death when these tissues are available. Real-time Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR) diagnostic assays (described below) are routinely performed on these samples to test for the presence of RNA from BTV and EHDV. During this study, we tested for the possible detection of RNA from BTV and EHDV through time in long bones of carcasses of deer that were found positive by the below criteria.

In 2013, samples were collected from five white-tailed deer with HD as a pathologically determined cause of death. BTV infection was confirmed by RT-qPCR of spleen samples. In 2015, samples were collected from three white-tailed deer with HD as a pathologically determined cause of death and EHDV infection was confirmed by RT-qPCR. All spleen and bone marrow samples collected at necropsy within 24 hours of death, or day 0, were tested using RT-qPCR assays to detect BTV and EHDV using the methods described below. The deer carcasses were then labeled and placed in a secure chain-link enclosure for sequential harvesting of bone marrow. The enclosures were

placed in a wooded area approximately 0.5 km away from the deer pens. Sample collection was performed by sharp dissection of tissues surrounding long bones, and then aseptically cleaned long-handled tree pruners were used to crack open the bone. A different leg bone was sampled for bone marrow at each collection time point. Disposable sterile spatulas were used to collect approximately 0.5-1.0g of bone marrow which was placed it into pre-labeled 2.0 mL Eppendorf vials. The vials were kept on wet ice and transported back to the lab where they were maintained at 2° C until testing. Bone marrow samples from the confirmed five BTV infected deer were taken weekly for 4 weeks and then sampled again at 8, 12, and 16 weeks respectively if testable marrow was still available. For the confirmed three EHDV infected deer, bone marrow was collected at 0, 2, 4, 6, 8, and 12 weeks post-mortem.

For the five BTV confirmed deer, bone marrow and spleen samples were split into duplicate subsamples for the RT-qPCR analysis. One subsample was submitted to Texas A & M Medical and Veterinary Diagnostic Laboratory (TVMDL) for multiplex RT-qPCR analysis and the other subsample was assayed at Louisiana State University. The RT-qPCR was conducted in both labs to compare results and affirm testing methodology. For the three EHDV confirmed deer, the RT-qPCR assays were conducted at Louisiana State University using the established protocols described below.

- PCR Diagnostics

Homogenates were prepared by crushing 0.10-0.20g of bone marrow or spleen in a 2.0mL Eppendorf vial using a plastic pestle and a pellet pestle motor (model #749540). Then, 900 µL of medium consisting of Hanks M-199 salts, 0.05 M Tris, pH 7.6, 1% bovine serum albumin, 0.35g/L sodium bicarbonate, 100 U/mL penicillin, 100ug/mL streptomycin, 1ug/mL Fungizone was added to each vial. Viral RNA was isolated from

the homogenate (50 µl tissue homogenate; 10% w/v in 1X phosphate buffered saline) using the KingFisher96 automated particle processor and the MagMAX™-96 Viral RNA Isolation Kit, as previously described (Schroeder et al. 2013).

Multiplex real-time reverse transcription PCR (RT-qPCR) for BTV and EHDV utilized the PathID Multiplex OneStep kit from Applied Biosystems/Life Technologies. Master mix preparation and cycling conditions were as previously described (Schroeder et al. 2013). The primer sets that were used in RT-qPCR assays are listed in Table 5.1. Samples were considered BTV positive when Ct values were less than 35 and less than 40 for EHDV.

Serotype was determined by reverse transcription and amplification of Seg-2 of BTV and EHDV. A single-tube reaction containing the SuperScriptTM III one-step RT-PCR system (Invitrogen) and high fidelity platinum Taq was used with the RNA template previously extracted from spleen or bone marrow samples shown to be positive via the RT-qPCR analysis; each primer set was used separately per reaction. The primer-template was heated to 95 ˚C for 3 min to denature the viral dsRNA, followed by immediate cooling on ice, and addition of the reaction mix. Amplification of segment-2 was carried out in 50 µL reaction volumes as described in Maan et al. (2012). The cDNA bands were stained with SYBERsafe (Invitrogen) and visualized under UV light. Samples containing bands of the correct size were considered positive (1349bp for BTV-10, 1613bp for BTV-12, 389bp for EHDV1, 246bp for EHDV2 and 500bp for EHDV6). The primer sets that were used in serotype assays are listed in Table 1.

- Cell Culture Assays

To determine if BTV/EHDV remained viable in bone marrow samples postmortem, tissues from 8 white-tailed deer that died from BTV/EHDV infection were

Table 5.1. Primers and probes for BTV and EHDV used in RT-qPCR and for traditional RT-PCR gel-based serotype assays.

Primers ID	Sequence (5′–3′) and probe reporter dye	Target Region	Amplicon size
	Bluetongue Virus RT-qPCR		
BTV_F	TGGAYAAAGCGATGTCAAA		
BTV_R	ACATCATCACGAAACGCTTC	NSP3	96bp
BTV_probe	FAM-ARGCTGCATTCGCATCGTACGC-BHQ1		
	Epizootic Hemorrhagic Disease Virus RT-qPCR		
EHD_F	ACWGGVATCATGTTTGAGCT		
EHD_R	TTCATAACCGCACCTTCATC	NSP1	110bp
EHD_probe1	VIC-TCATCACACATCGGC-MGB		
EHD_probe2	VIC-TCTCGGCATATGCGAGC-MGB		
	BTV and EHDV Serotype Assays		
BTV-10F	TTCGGCGTGTTATGCTAACTTC	VP2	1349bp
BTV-10R	CCACTGCGCCCAACCTT		

(table cont'd)

Primers ID	Sequence (5′–3′) and probe reporter dye	Target Region	Amplicon Size
BTV-12F	TYTGRGATCACAAYATAGAYRTG	VP2	1613bp
BTV-12R	GTACTCCGTCTTTGGAAGATG		
EHDV1-F	AATAGGCGATGTTGATCGACATC	VP2	389bp
EHDV1-R	TTCGAAACCTCGCTTGCAT		
EHDV2-F	TGGTGAAAATACGGTGGTATATAACC	VP2	246bp
EHDV2-R	GTTCAAATTCATCTGGGCTCATACT		
EHDV6-F	ATAACGAACAGGGAGCCKTATAAAA	VP2	500bp
EHDV6-R	CCAAACTTCTCAGTAGCATACAACAT		

used. In 2016, one white-tailed deer died of BTV infection, and in 2017, two deer died from BTV and five died from EHDV infection. Bone marrow and spleen samples were taken as described above on day 0, and then bone marrow was collected at 3, 7 and 10 days postmortem; RT-qPCR was performed to test for viral nucleic acid and virus isolation was attempted using cell culture techniques described below.

Attempts to isolate BTV and EHDV via cell culture were made using bone marrow samples that were RT-qPCR positive from eight white-tailed deer carcasses (three positive for BTV and five positive for EHDV). Virus isolation was attempted utilizing two different cell lines: baby hamster kidney cells (BHK) and Vero cells. These two cell lines have been shown to be susceptible to *Orbivirus* infection (Wechsler and McHolland 1988). The cell lines were maintained with media made from 2% antibiotic-antimycotic (p/s/f), 10% fetal bovine serum (FBS), and 88% Medium 199 (1x). Using T-75 cell culture flasks, 100 µL of sonicated homogenate from the bone marrow samples were mixed with 10mL of 5% FBS cell culture media and then inoculated into a confluent T-75 flask containing Vero or BHK cells. Cultures were then incubated in a humidified 5% CO_2 atmosphere at 37° C and monitored daily for CPE, or cytopathic effect (Ammerman et al. 2008). If there was no CPE after 8-10 days, cells were harvested and 100 µL of the cell suspension was inoculated into an additional Vero/BHK cell culture for a second passage. Cultures were considered negative if CPE was not observed after the second passage (Ruder et al. 2012); RT-qPCR was conducted on the cell culture samples to confirm that the samples were negative for BTV/EHDV.

5.3 Results

In October and November 2013, the spleen and bone marrow of the five white-tailed deer that died with clinical signs of HD were RT-qPCR positive for BTV at day 0.

Of the five, three deer were positive for BTV-12 and two for BTV-10 (Tables 5.2 and 5.3). Spleen and bone marrow from the hunter-killed deer were RT-qPCR negative on day 0 and the bone marrow samples were also negative at 2 and 4 weeks post mortem. All bone marrow samples that were positive on day 0 remained BTV-positive throughout the experiment. The Ct values of the bone marrow from RT-qPCR ranged from 16.10 - 34.80 and for spleen 18.50 – 29.60. Although the Ct values varied slightly between laboratories (Table 5.2), the conclusive results from the RT-qPCR assays were equivalent from TVMDL and LSU; all bone marrow samples tested from 2013 had Ct values less than 35 and were considered BTV positive.

During September through November in 2015, three white-tailed deer showed classical clinical signs for HD and subsequently died. All samples from day 0 for spleen and bone marrow were RT-qPCR positive for EHDV (Table 5.3). The Ct values for spleen ranged from 22.45-27.42 and for bone marrow 24.24-37.86. All bone marrow tested positive for EHDV except for two of three samples at 12 weeks post mortem. All three bone marrow samples from the deer were serotyped and determined to be EHDV-2.

In 2016 and 2017, three deer died with active BTV infections and five deer died with active EHDV infections. For all deer, BTV or EHDV was isolated from bone marrow samples collected on day 0 using cell culture technique. No virus isolation was achieved from bone marrow samples past day 0, while RT-qPCR results for BTV and EHDV in the bone marrow were positive up to 10 days post-mortem (Table 5.4 and 5.5). The RT-qPCR assay also was conducted on the cell culture samples, and all samples were negative except the samples tested on day 0.

Table 5.2. Spleen and bone marrow RT-qPCR results reported as Ct values for the tissues of five white-tailed deer that died of BTV infection in 2013 at the Bob R. Jones Idlewild Research Station in Clinton, Louisiana. Ct values less than 35 are positive for BTV.(Louisiana State University /Texas A and M Veterinary Medical Diagnostic Laboratory)

						Ct Value of Bone Marrow							
ID	Age	Sex	Death Date	Type	Spleen Ct day 0	day 0	day 7	day 14	day 21	day 28	day 56	day 84	day 112
BN-53	2	F	7-Oct-13	12	21.3/18.5	NA[a]/27.3	NA/24.4	NA/NA	29.75/25.7	30.4/30.9	30.7/27.9	29.5/34.7	NA/NA
R-39	3	F	14-Oct-13	10	16.9/20.6	35.0/29.6	34.81/28.5	27.7/25.7	27.92/28.1	33.5/29.6	33.7/32.5	30.2/34.4	30.6/31.4
Y-15	0.5	F	14-Oct-13	12	29.6/19.2	27.4/22.3	33.74/24.5	31.0/24.4	27.44/27.3	NA/NA	NA/NA	NA/NA	NA/NA
P-88	2.5	F	25-Oct-13	12	29.4/24.4	27.9/16.1	27.74/20.8	27.4/21.5	31.73/22.3	30.7/26.2	29.6/26.7	29.7/27.7	NA/NA
Y-75	0.4	M	16-Nov-13	10	29.6/18.7	27.9/24.7	26.22/27.5	27.7/27.5	29.6/26.4	29.6/26.1	29.4/34.2	29.6/25.2	30.3/24.7

[a] NA= tissues not available for testing

Table 5.3. Ct values from RT-qPCR for bluetongue virus (BTV) and cell culture results noted by presence of CPE (cytopathic effect) from bone marrow samples of white-tailed deer ranging from day 0 to day 10 PM (post-mortem) collected at the Bob R. Jones Idlewild Research Station in 2016-2017.

Deer ID	Days Post Mortem	Bone Marrow Ct	BHK CPE	BHK Cell Culture Ct	Vero CPE	Vero Cell Culture Ct
Y-81	0	28.63	Yes	32.16	Yes	27.76
	3	32.13	No	>35 [a]	No	>35
	7	29.36	No	>35	No	>35
	10	27.37	No	>35	No	>35
0-72	0	29.36	Yes	30.61	Yes	31.23
	3	31.35	No	>35	No	>35
	7	32.49	No	>35	No	>35
	10	25.37	No	>35	No	>35
G-90	0	31.13	Yes	28.94	Yes	29.43
	3	21.15	No	>35	No	>35
	7	19.50	No	>35	No	>35
	10	21.51	No	>35	No	>35

[a] >35 = negative result for BTV

5.4 Discussion

This study was the first to show that BTV can be detected from bone marrow samples collected from white-tailed deer carcasses for up to four months postmortem and EHDV for up to 3 months postmortem. The results of this study show that these methods can be successfully implemented to determine if active virus was present at the time of a deer's death. This information combined with historical herd data and observations can help determine possible causes of mortalities retrospectively. Considering the known environmental stability of these viruses (Kienzle et al. 2017), this would be expected if there are no RNA lytic factors that occur in the intact bone marrow. Our results do not

Table 5.4. Ct values of bone marrow from RT-qPCR for epizootic hemorrhagic disease virus (EHDV) and cell culture results noted by presence of CPE (cytopathic effect) from bone marrow samples of white-tailed deer ranging from day 0 to day 10 PM (post-mortem) collected at the Idlewild Research Station in 2017.

Deer ID	Days Post Mortem	Bone Marrow Ct	BHK CPE	BHK Cell Culture Ct	Vero CPE	Vero Cell Culture Ct
0-19	0	27.24	Yes	29.41	Yes	31.45
	3	25.25	No	>40 [a]	No	>40
	7	34.34	No	>40	No	>40
	10	26.81	No	>40	No	>40
0-40	0	33.63	Yes	31.32	Yes	35.64
	3	27.12	No	>40	No	>40
	7	17.37	No	>40	No	>40
	10	24.48	No	>40	No	>40
0-5	0	24.36	Yes	28.51	Yes	33.23
	3	25.37	No	>40	No	>40
	7	22.74	No	>40	No	>40
	10	27.83	No	>40	No	>40
PK-24	0	34.46	Yes	34.54	Yes	31.41
	3	28.10	No	>40	No	>40
	7	18.58	No	>40	No	>40
	10	27.97	No	>40	No	>40
G-73	0	17.63	Yes	30.30	Yes	33.22
	3	22.12	No	>40	No	>40
	7	22.27	No	>40	No	>40
	10	31.45	No	>40	No	>40

discount the potential for detecting the RNA of these viruses for much longer periods than 3-4 months, but practically this is an adequate time frame to examine deer mortalities retrospectively. We did not record meteorological data, but the carcasses were exposed to typical elements in Louisiana during late summer. Carcasses of deer are often discovered by hunters scouting for deer in a period that falls into the time frame following typical Orbivirus transmission periods in late summer and early fall. The results of this study are important because the methods described can serve as an excellent tool to confirm circulating BTV/EHDV among a white-tailed deer

Table 5.5. Ct values of bone marrow from RT-qPCR for epizootic hemorrhagic disease virus (EHDV) and cell culture results noted by presence of CPE (cytopathic effect) from bone marrow samples of white-tailed deer ranging from day 0 to day 10 PM (post-mortem) collected at the Idlewild Research Station in 2017.

Deer ID	Days PM	Bone Marrow Ct	BHK CPE	BHK Cell Culture Ct	Vero CPE	Vero Cell Culture Ct
0-19	0	27.24	Yes	29.41	Yes	31.45
	3	25.25	No	>40 [a]	No	>40
	7	34.34	No	>40	No	>40
	10	26.81	No	>40	No	>40
0-40	0	33.63	Yes	31.32	Yes	35.64
	3	27.12	No	>40	No	>40
	7	17.37	No	>40	No	>40
	10	24.48	No	>40	No	>40
0-5	0	24.36	Yes	28.51	Yes	33.23
	3	25.37	No	>40	No	>40
	7	22.74	No	>40	No	>40
	10	27.83	No	>40	No	>40
PK-24	0	34.46	Yes	34.54	Yes	31.41
	3	28.10	No	>40	No	>40
	7	18.58	No	>40	No	>40
	10	27.97	No	>40	No	>40
G-73	0	17.63	Yes	30.30	Yes	33.22
	3	22.12	No	>40	No	>40
	7	22.27	No	>40	No	>40
	10	31.45	No	>40	No	>40

[a] >40 = negative result for EHDV

population during a time of high mortalities. Previously, white-tailed deer carcasses that were found near water were assumed to be the result of a hemorrhagic disease epizootic. These assumptions are generally made because deer affected with HD can have high fever in excess of 105°F, become dehydrated, and move towards water prior to death. Confirming that epizootics are associated with BTV or EHDV can at least rule out other important pathogens, domestic or foreign, which would require further investigation. There are important diseases that all wildlife personnel should be aware of that clinically could mimic HD, such as Malignant Catarral Fever, Heartwater Disease and Chronic

Wasting Disease (Mackintosh 1998). These diseases should be investigated anytime or at least when there are deer mortalities that are RT-qPCR negative for BTV/EHDV and that are not within the usual season of transmission.

This study was also the first to show that BTV/EHDV serotypes can be determined from white-tailed deer bone marrow collected postmortem. Determination of serotype is vitally important especially when tracking large scale epizootics of HD. For the widespread U.S. epizootic of 2012, it is unknown if environmental conditions favored vector population numbers associated with increased transmission of multiple serotypes of BTV or EHDV, or whether there was a rapid spread of strains of virus via alternative routes. If HD vaccines are to be developed in the future, it will be imperative to know the history of transmission patterns in varied geographical areas. Testing the bone marrow of deer carcasses could be used to help establish those patterns. Because effective BTV/EHDV vaccines are serotype specific, the most prevalent serotypes within a region would need to be known to deliver maximum efficacy.

Deer farming has increased, especially in the southern U.S., and hemorrhagic disease can be very detrimental for captive white-tailed deer herds. Mortality rates can reach over 80% in naive herds and the economic losses for a single deer farmer can be devastating. When an outbreak of HD occurs, many deer farmers are not prepared to collect the appropriate tissues for testing or fresh samples are unavailable because of scavenging. With the information generated from this study, farmers, herd managers, biologists and veterinarians can determine which virus and serotype was circulating within a herd and thus make management decisions to reduce transmission or develop efficacious vaccines.

Although BTV/EHDV can be detected in the bone marrow of white-tailed deer for extended periods after death using RT-qPCR methods described, our results showed that viable viruses could not be isolated after 24 hours of death, under normal post-mortem conditions. In conclusion, the dsRNA of EHDV and BTV can be detected in white-tailed deer bone marrow for up to 3 to 4 months postmortem under normal environmental conditions in south Louisiana, but the detection of the RNA of these viruses was not equivalent to virus viability.

SUMMARY AND CONCLUSIONS

In our study, the CDC black light traps captured the same or additional species of *Culicoides* than any other trap type. We showed that BTV MIR is higher in infected *Culicoides* species captured in CDC traps without light than with light. However, CDC traps with light overall captured significantly more specimens of *Culicoides* and should be used in addition to traps without light when conducting biting midge surveillance. For EHDV we did not see a higher MIR in the traps without light and can conclude that there is a difference in the MIR in EHDV infected flies vs MIR of BTV infected flies in different traps with or without light. More studies are needed to determine why these differences exist in midges infected with BTV versus midges infected with EHDV.

When conducting *Culicoides* midge surveillance in an unknown area with orbivirus transmission, CDC traps with and without black light should be used to capture the most species/specimens of BTV/EHDV infected midges. Subsequently, to determine activity times of species of interest timed traps could be used and then more intensive studies can be conducted during those peak activity times using techniques of value to the objectives of the study. We found that the most active time for midges of *C. debilipalpis* and *C stellifer* is from 0600-0800h, near sunrise, and recommend this time period as the optimal time to spray insecticides to control the important vectors of BTV/EHDV in Louisiana.

Over 7 years of trapping *Culicoides* midges at the BJIRS, we observed some years the peaks of midge vector activity peaked at the same time as the confirmed deer deaths but in other years the peaks of activity were not obviously associated with deer deaths. The overall pattern of midge activity and deer deaths showed clear patterns of association of the suspected vector species with confirmed BTV and EHDV deaths. Therefore, it is

important to conduct these types of studies over multiple years to get the overall concept of midge activity versus transmission. Observed peaks of competent vector midge activity do not always correspond with BTV/EHDV transmission patterns and midge activity using light traps can vary nightly depending on abiotic factors such as moon phase, temperature, cloud cover, or wind. By combining all the data from the midges and dead deer over 7 years, we get a clear understanding of the positive correlation of midge abundance versus confirmed deer death on a temporal scale. The evidence in this study supports the idea that the 2 major vectors driving BTV transmission in Louisiana are *C. debilipalpis* and *C. stellifer*. Our results confirm that *C. venustus* and *C. stellifer* can be competent vectors for EHDV as concluded by McGregor et al. (2018). Future studies are needed to elevate their status as confirmed BTV/EHDV vectors. The establishment of laboratory colonies of suspected species is needed, which could then allow significant investment from the federal government to conduct research on important BTV/EHDV vector species other than *C. sonorensis*.

Although BTV/EHDV can be detected in the bone marrow of white-tailed deer for extended periods after death using RT-qPCR methods described, our results showed that viable viruses could not be isolated after 24 hours of death, under normal post-mortem conditions. In conclusion, the dsRNA of EHDV and BTV can be detected in white-tailed deer bone marrow for up to 3 to 4 months postmortem under normal environmental conditions in south Louisiana, but the detection of the RNA of these viruses was not equivalent to virus viability. This information will be a great tool for deer farmers and biologists to utilize during epizootics when the cause of deer deaths is unknown. By determining the virus and serotype in an area from bone marrow off of dead deer, researchers and farmers/biologists could implement vaccination plan which

may be the best method to prevent BT, EHD, and HD in white-tailed deer because spraying for control of midges has been largely unsuccessful.

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VITA

Michael Edward Becker was born to Edward and Kathleen Becker on April 2, 1982 in New Orleans, Louisiana. He received his bachelor of science degree majoring in biological sciences at Louisiana State University in December of 2004. In his last semester before graduation with his bachelor's in biology, he was employed as a student worker by Cole Younger in the Foil Lab in the Department of Entomology at LSU. Upon graduation with his B.A., he became a transient worker under Lane Foil and worked until January 2006 when he enrolled as a graduate student in the Department of Entomology at LSU. He was employed as a graduate assistant under Lane Foil working on a bluetongue virus project in south Louisiana. Michael received his master of science in entomology in August 2008, and was immediately hired as a full time research associate in Lane Foil's lab. Michael entered his PhD program in 2013 as a part time student where he remained a research associate and plans to graduate in May 2020 with his PhD in entomology.